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An investigation of 2D and 3D substrates for embryonic facial process culture

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September 2011

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Abstract

This work aims to highlight the differences between the use of 2D and 3D cell culture systems for the growth of samples of primary embryonic mesenchymal tissue derived from facial processes. In the developing embryo, these regions of tissue go on to form the cartilaginous template of the craniofacial skeleton. The tissue used in these experiments was derived from the frontonasal mass and mandible of chick embryos, which form parts of the upper and lower beak respectively. I have shown that explanted pieces of mesenchymal tissue from the frontonasal mass and the mandible of chick embryos display different cell behaviours in both traditional 2D cell culture and when grown in alvetex[®], a 3D cell culture system that uses a polymer scaffold, developed by Reinnervate Ltd. In 2D culture, as previously described in the literature, populations that expand from explanted frontonasal mass form sheets of cartilaginous material, whereas mandible cultures seem to expand into two different populations of cells, a sheet-like population as seen in frontonasal mass cultures as well as a population that is fibrous in appearance.

3D counterparts invaded and populated the alvetex[®], and again there appeared to be one population of cells expanding from frontonasal mass explants and two populations in the case of explanted mandible tissue. 3D populations had dramatically higher levels of Alcian blue staining (indicative of the presence of cartilaginous material) than 2D cultures, suggesting that alvetex[®] provides a more permissive environment for the production of cartilage than traditional 2D substrates used in cell and tissue culture. It was also shown that the alvetex[®] appears to separate the two different populations of cells observed in mandible cultures, in that the two populations occupy the internal surface of the polymer at 7 days culture, but by 14 days one population remains within the internal surface whilst the other has grown along the top surface of the polymer.

These results suggest that alvetex[®] provides an environment that is suitable for the growth of primary embryonic tissue and may be suitable for the culture of other samples of primary tissue from which cell lines that have been established in a 3D environment could be derived.

Introduction

The craniofacial skeleton is derived from a population of cells in the developing embryo known as neural crest. Neural crest is a multipotent population of cells that arises along the dorsal surface of the neural tube in a developing embryo. Populations of neural crest cells follow discrete migratory pathways to contribute to a variety of tissues in the developing embryo, including neurons, glia, smooth muscle, cartilage and bone (reviewed Tucker 2004).

Cranial neural crest cells occupy the most anterior region of the neural tube and contribute to the craniofacial skeleton and its associated connective tissues and the cranial ganglia that innervate the face. This specific cell population migrates from the prosencephalon, mesencephalon and hindbrain of the embryonic brain to contribute mesenchymal tissues to the majority of the facial skeleton and branchial arches (Osumiyamashita et al. 1994). There are many documented mutations that affect the migration of cranial neural crest and can cause abnormalities in the development of the craniofacial skeleton, the effects ranging from mild to severe facial deformities.

The craniofacial skeleton begins as a cartilaginous template that develops from mesenchymal tissue, which eventually ossifies to become bone.

(Hall and Miyake 2000) split the process of skeletogenesis into four primary phases; the migration of cells to the site of skeletogenesis, the epithelial-mesenchymal interactions, cell condensation, and overt differentiation.

Chondrogenesis is initiated when cell condensation occurs and leads to differentiation of mesenchymal chondrogenic pre-cursor cells to chondrocytes.

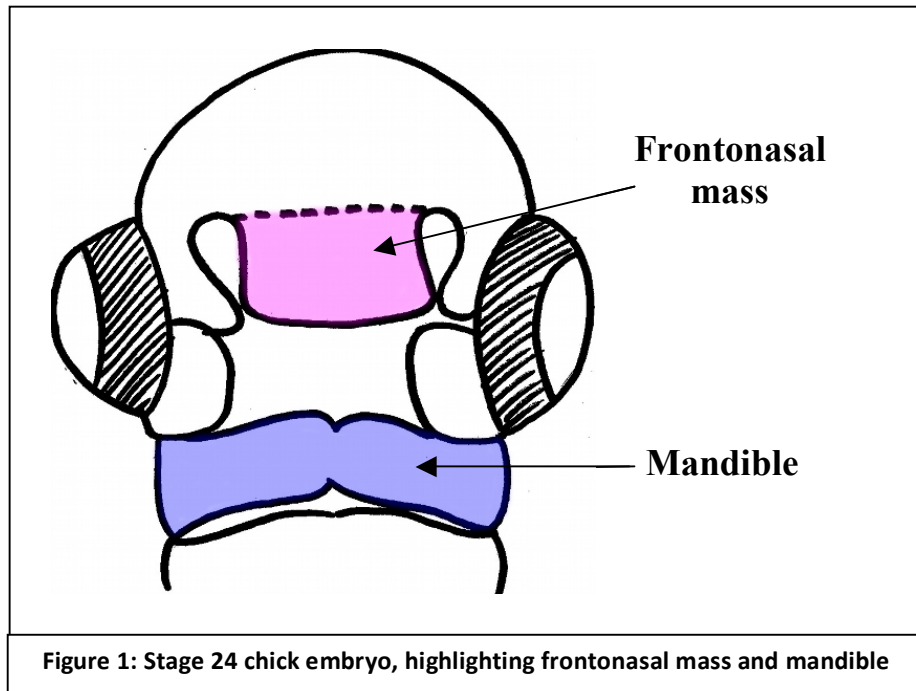
Cell condensation is seen *in vivo* as an aggregation of cells and occurs prior to any overt chondrogenesis. It is thought that TGF-beta and fibronectin regulate N-CAM, a cell adhesion molecule, which initiates this aggregation of cells, resulting in a condensation. There is an increase in the expression of Pax genes, known to be involved in epithelial-mesenchymal interactions, an important process of reciprocal signalling that promotes the outgrowth of cartilage in condensations in the developing embryo. Condensations begin to differentiate and become chondrogenic as there is a decrease in cell adhesion molecules, such as N-CAM,

and genes associated with proliferation, such as fibronectin, alongside and increase in genes associated with differentiation, such as BMPs and FGFs. The expression of these adhesion molecules was shown to increase during cell aggregation and then became undetectable in hypertrophic chondrocytes (Tavella et al. 1994).

As the production of cartilaginous extracellular matrix commences, there is a decrease in cell density. The cartilage matrix itself is composed of macromolecules that include collagens II, IX, XI and aggrecan (Kulyk et al. 2000). The expression of genetic markers associated with chondrogenesis has been well characterised, and in particular the roles and interactions of *Sox9* and *Col2a1*. *Sox9* is a HMG-domain transcription factor, a well known regulator of a major constituent of cartilaginous matrix, collagen type II and the first specific marker of chondrogenesis. It has been shown that *Sox9* directly regulates the expression of *Col2a1* by binding to a regulatory domain in the first intron of the *Col2a1* gene (Bell et al. 1997).

Using in situ hybridisation (Wright et al. 1995; Healy et al. 1999) it has been shown that *Sox9* is expressed at sites of chondrogenesis and its expression is linked to cartilage production in hyaline cartilage through the alteration of expression of BMPs, growth factors that are expressed upstream of *Sox9*. This was explored in the chick limb, a system that is often compared to the development of the craniofacial system. By inducing exogenous expression of BMP2, expression of *Sox9* subsequently increased and ectopic cartilage was produced. Conversely, by repressing the expression of BMP2 with the antagonist *Noggin*, there was a decrease in the expression of *Sox9* in the developing digits of the chick embryos.

In avian embryos the cartilage and bone that forms the craniofacial skeleton are derived from four facial processes; the frontonasal mass, the mandible, the maxillae and the lateral nasal process. These processes arise from cartilage condensations and their subsequent outgrowth and fusion form the facial skeleton. The experiments described in this series of experiments focus on two of these processes, the frontonasal mass and the mandible.



The frontonasal mass forms part of the upper beak and primary palate in avian embryos, while the mandible forms the rods of Meckel's cartilage in the lower beak. The structures that are formed by these two facial processes are very different in morphology and yet they are both derived from cranial neural crest derived mesenchyme.

Micromass cultures have been used as a tool with which to investigate the 'chondrogenic potential of mesenchymal cell populations' (Wedden et al. 1986) and have previously been used to investigate the properties of mesenchyme derived from different facial processes. Micromass cultures use droplets of cell suspensions created from mesenchymal tissue to induce chondrogenesis. The droplets used in this method are of very high cell density in order to imitate the conditions of mesenchymal condensation, inducing differentiation of cells to chondrocytes within the suspension.

Tissue was obtained from the facial processes of stage 24 (Hamburger and Hamilton 1951) chick embryos and enzymatic digestion was used to dissociate the tissue and create a cell suspension. Samples were taken from the frontonasal mass, mandible and maxillae. This cell suspension was then applied as a cell droplet to Petri dishes, allowed to adhere to the substrate below and cultured. The resulting cultures produced cartilaginous patterns that are characteristic for each of the facial processes that were investigated. The

frontonasal mass produced a flat sheet of cartilaginous material, the mandible produced nodular cartilage and the maxillae produced little to no cartilage. Considering that these facial processes produce very different cartilaginous structures *in vivo*, these results indicate that these two populations are intrinsically different at the stage at which the micromass cultures are set up given that their cell behaviours are very different despite being grown in identical environments *in vitro*.

This difference in cell behaviour indicates that the two tissues have received different developmental signals or at different stages of chondrogenesis at the time the tissue is explanted from the embryo.

The aim of my work is to further investigate the behaviour of cells that are derived from these two sources of tissue by translating the micromass culture method into a novel 3D cell culture environment.

While 2D cell culture technology has been enormously beneficial in our understanding of the growth and differentiation of different cell types, it is not an accurate representation of the native environment experienced by cells *in vivo*. The cells are removed from a three dimensional tissue environment and grown on flat two dimensional substrate, an environment that alters their cellular architecture and can result in aberrant cell behaviour that would not normally be observed *in vivo* (Schutte et al. 2009). While conventional cell culture in 2D plasticware is convenient, it limits the conclusions that can be drawn from the results it generates.

While there are many different methods employed to create 3D scaffolds for the culture of cartilaginous tissue (Tortelli and Cancedda 2009) and many teams have developed biodegradable 3D scaffolds (e.g. Brun et al. 1999) that are designed as *in vivo* implants, there is little in the way of scaffolds that are designed for routine 3D cell culture assays. Evidence from biodegradable scaffolds suggest that they provide a much more permissive environment for the culture of mature chondrocytes in comparison to 2D cultures, with ultrastructural observations of the cells showing that they maintained their chondrogenic organisation and phenotype, and produced a cartilage-like extracellular matrix. Brun et al also noted the risk of growing chondrocytes in 2D

culture was that cells often de-differentiated in 2D monolayers and took on a more fibroblastic appearance, a problem that seemed to be circumvented by 3D cell culture in these biodegradable scaffolds.

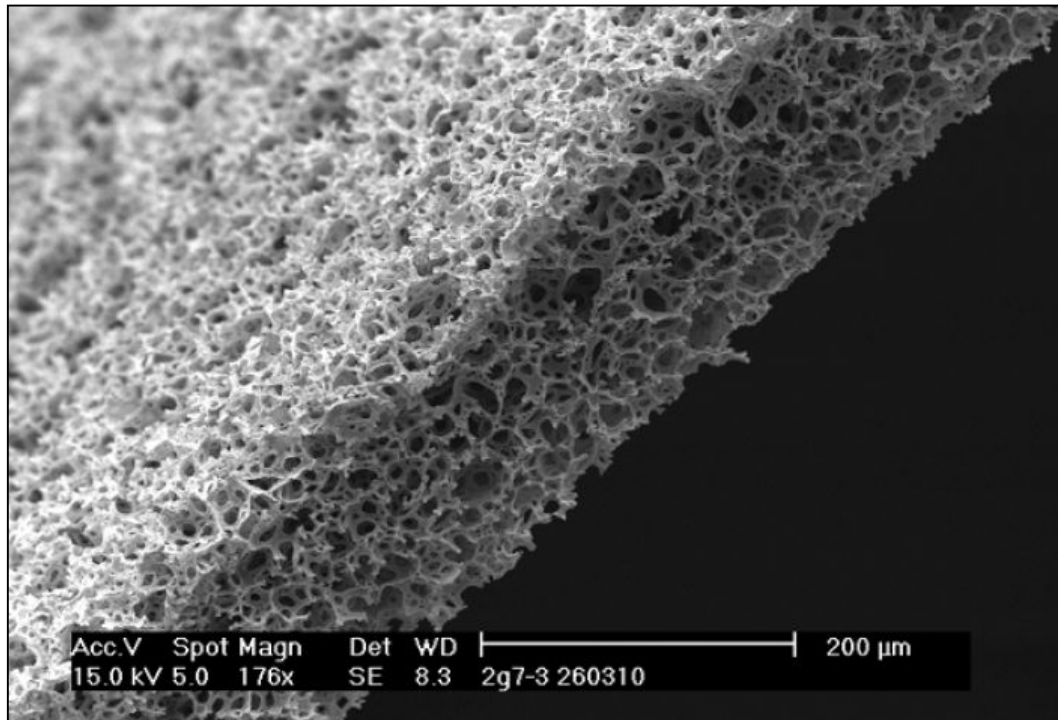


Figure 2: alvetex® scaffold under high magnification (image property of Reinnervate Ltd.)

Alvetex® is a 3D scaffold produced by the polymerisation of high internal phase emulsions (HIPEs) to create PolyHIPEs (Bokhari et al. 2007). PolyHIPEs are relatively homogenous, have a low density and open cellular construction with the possibility to alter porosity and void interconnections. This allows the invasion and proliferation of cells within the polymer scaffold. Scaffolds are cut from a PolyHIPE monolith to produce discs that are 200μm thick, meaning that the scaffold is thin enough to allow sufficient diffusion of nutrients and waste products but strong enough to withstand manual handling. The scaffold has been previously tested with cell lines such as HepG2, where 3D samples were shown to a population of cells with normal cell architecture (Schutte et al. 2009). The cells grow in 2D formed flat, disorganised structures, that when compared to their 3D counterparts had fewer of the morphological structures that are associated with *in vivo* liver tissue. The cells grown in 3D were metabolically active, producing a significantly higher volume of albumin than their 2D counterparts and also showing evidence of the formation of bile canaliculi.

Using alvetex® 3D cell culture technology with a chondrogenic micromass system would present a cell culture environment more in line with the environment that these cells would encounter *in vivo*. As it has been demonstrated previously using a well established and characterised cell line, it will be interesting to see if the same is true of primary samples of embryonic chondrogenic tissue, and if the cell behaviour observed in a 3D scaffold is more *in vivo*-like than that observed in traditional 2D cell culture. Culturing primary tissue samples in 3D in a matrix that provides a consistently reproducible environment would allow the derivation of cell lines that would have never been cultured in a 2D environment, and hence would not have a genome that has been altered to suit growth in a flat two dimensional environment.

The aim of alvetex® is to provide an inert three dimensional substrate that can be used for routine 3D cell culture. If the micromass method can be translated into this 3D cell culture technology, it would allow the creation of chondrogenic tissue that is more *in vivo*-like. This would mean that any experimental assays that are applied to these chondrogenic cultures are more likely to produce results that more accurately reflect results that would be achieved if the same experiment was applied *in vivo*.

This series of experiments aims to investigate the differences between the behaviours of cells obtained from primary samples of tissue of avian facial processes in traditional 2D cell culture plasticware and alvetex® 3D cell culture technology. The majority of cells that are grown in 3D scaffolds are cell lines that have first been cultured in a 2D environment before being transferred into 3D cell culture. This is a novel experiment, as I have used cells that are obtained directly from 3D samples of avian tissue and culturing them in a 3D environment.

I have developed a protocol that allows the culture of primary avian mesenchymal tissue in a synthetic 3D cell culture environment. This protocol has been used to assess the differences in cell behaviour between different tissue types, frontonasal mass and mandible, and different culture environments, two dimensional and three dimensional. The two tissue types, frontonasal mass and mandible were chosen as earlier micromass work showed that they displayed

very different cell behaviours *in vitro* when cultured in 2D, so it will be interesting to see if the same holds true for equivalent 3D cultures.

The results demonstrate how the different tissue types invade the polymer scaffold, the different patterns of growth that are exhibited between 2D and 3D cultures and the affect of a 3D scaffold on the production of cartilaginous matrix. It also highlights the potential for alvetex® as a sorting mechanism for cell populations obtained from primary tissue and provides the basis for a new cell tracking system that can be applied to cultures grown within an opaque 3D substrate.

Materials and Methods

Cell culture equipment

Cells being cultured in 2D were cultured on the bottom of standard 12 well plate cell culture plasticware (Grenier Bio-One) and cells cultured in 3D were cultured on a 3D polymer scaffold, suspended in cell culture using a custom well clip (Reinnervate Ltd.), see Figure 3.



Figure 3: Reinnervate Ltd. 12 well alvetex® 3D cell culture dish – A normal 12 well cell culture plate modified for 3D cell culture purposes. Micromass high density droplets or explanted mesenchymal tissue pieces were placed at the centre of polymer discs (seen here as white discs at the bottom of culture well). Discs are held in place by plastic clips. (Image courtesy of Reinnervate)

Alvetex® 3D cell culture plates used in this series of experiments were either manually prepared using standard 12 well cell culture plates, alvetex® derived from second generation production batch number 36, and manually placing plastic clips over the polymer discs, or alternatively pre-prepared commercial packs of 12 well plates with alvetex® already clipped into place were used. All plasticware, alvetex® discs and well clips were sterilised by progression through an ethanol series to Dulbecco's PBS (Sigma-Aldrich).

Cell culture medium

Medium contains MEM α (Invitrogen - Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1% penicillin/streptomycin (Lonza – BioWhittaker) and 1% l-glutamine (Lonza).

Preparation of micromass cultures

Tissue samples were obtained from stage 24 (Hamburger and Hamilton 1951) White Leghorn chicks obtained from a commercial source (PD Hook Hatchery, Dalton). Embryos were removed from the yolk and washed in Tyrode's salt solution, an isotonic tissue culture holding solution, supplemented with 1% penicillin/streptomycin. Micromass cultures were then established for samples. Frontonasal mass and mandible tissue explants were dissected from the embryos using flame-sharpened tungsten needles, and samples pooled into either groups of frontonasal mass or mandible.

The samples were dissociated by enzymatic action using 0.1% trypsin (Sigma-Aldrich) and incubated at 37°C for 20 mins. The tissue samples were then manually dissociated using a pipette and pelleted by centrifugation. The supernatant was removed and resuspended in 1ml of cell culture medium, then passed through a Nitex 20 cell sieve to remove clumps of cell debris. A cell count was then made using a haemocytometer and trypan blue exclusion (Sigma-Aldrich) and the resulting cell counts were used to adjust the number of cells in solution to 2×10^7 cells/ml.

For 2D cultures, 20 μ l droplets were inoculated onto the bottom of sterilised plasticware. For 3D cultures, 20 μ l droplets were inoculated onto the centre of the suspended polymer discs. Plasticware and polymer discs were sterilised either by passage through an ethanol series through to Dulbecco's PBS (Sigma-Aldrich) or by oxygen plasma treatment.

Inoculated plates were placed in an incubator at 37°C and 5% CO₂ for a minimum of 1 hour to allow cell adhesion to the substrate. 1ml of culture medium was then added and cultures incubated for upto 14 days, with culture medium being changed every 48 hours.

Preparation of mesenchymal explants

Tissue samples were obtained from stage 24 chick embryos, with the embryos being removed and dissected as described above. The frontonasal mass and mandible samples were put into either pancreatin or dispase (Sigma-Aldrich) and incubated at 37°C for 10 and 15 minutes respectively. Tungsten needles were then used to separate the epithelium from the mesenchyme and the epithelium was discarded.

The samples were then transferred either to suspended polymer discs for 3D culture or alternatively transferred to the bottom of a 12 well cell culture plate.

Fixation for Alcian blue staining

After incubation, cultures were washed in Dulbecco's PBS and fixed with either 10% formalin (Sigma-Aldrich), Serra's fixative (10% glacial acetic acid, 30% formalin, 60% 100% EtOH) for 20 minutes. For Alcian staining of whole cultures, the samples were washed in acetic acid, then bathed in Alcian blue (R. A. Lamb) dissolved in 3% aqueous acetic acid at pH7.5 overnight. Samples were then washed again in 3% aqueous acetic acid, viewed under a dissection microscope (Zeiss Stemi SV11), photographed (Spot Idea Camera) and the photographs processed (Spot Basic Software). Alcian blue staining indicates the presence of glycosaminoglycans characteristic of cartilage matrix production.

Histological staining with crystal violet

The histological stain crystal violet was used to visualise cells in both 2D and 3D cultures. After incubation, cultures were washed in Dulbecco's PBS and fixed in 10% formalin for 20 minutes. 1ml 0.05% crystal violet (dissolved in distilled water and filtered to remove excess powder) was then added to a cell culture well for 10-15 mins, removed and the cultures washed in Dulbecco's PBS before being photographed under a dissection microscope.

Histological analysis with Mayer's Haematoxylin and Eosin and Masson's Trichrome

Samples were washed in Dulbecco's PBS , and those to be analysed with H&E were fixed in 10% formalin. Samples to be analysed with Masson's Trichrome were fixed in Bouin's fixative (for 100ml: 75ml saturated picric acid, 25ml 40% formaldehyde and 5ml glacial acetic acid). All samples were then embedded in paraffin wax and sectioned at 10µm on a microtome. Sections were mounted on histobond coated slides, then stained with H&E and allowed to dry overnight. Slides were then examined under a reflected inverted light microscope (Leica DM500) for the presence of cells, photographed (Leica ICC50) and brightness and contrasted adjusted with LAS EZ (version 2.0.0.292) software.

Quantification by Alcian absorbance

The relative presence of cartilage present in cultured samples was indicated by measuring absorbance values at 600nm for samples stained with Alcian blue, with higher absorbance values indicating a higher proportion of cartilage present. Samples stained with Alcian blue were bathed in 300µl of Guanidine HCl at 4M overnight to extract the dye. 200µl samples were then taken from each well, put into a 96 well plate and had absorbance values read by a BioTek ELx800 plate reader. 200µl of Guanidine HCl was used as a blank control to account for background absorbance.

Visualisation of cell migration from explants

Tissue explants were labelled with the long-chain carbocyanine fluorescent membrane probe Cell Tracker CM Dil (Invitrogen) in order to observe the migration of cells from the original pieces of explanted tissue. At the stage of explant preparation when the epithelium is enzymatically removed from the mesenchymal tissue, the molecular probe is added to the enzyme digest mix. Stock solutions of Dil were prepared by adding 50µl of DMSO to 50µg of Dil solid crystals to create a 1mg/ml stock solution. 1µl was then added to 1ml of the enzyme digest mix to create a 1µM concentration of Dil.

The explant preparation protocol is then continued as normal from this point.

Photomicrographs of the cells were then taken at Day 1, 3, 5, and 7 on an inverted fluorescence microscope to monitor the migration of labelled cells in both 2D and 3D.

Measurement of cell neighbour proximity as a representation of cell density

A representation of cell density was created by measuring the distance between a cell and its closest neighbouring cell in 3D sections of tissue explant cultures using NIH ImageJ (version 1.44o) software. Photographs of whole sections of 3D cultures were loaded into the software and each individual cell was labelled with a dot which logged the XY co-ordinates of that cell within the photograph. An individual 'cell' which was logged in this manner was deemed to be a cell body with a visible cell nucleus, where the nucleus was chosen as the location for the labelling dot for that cell. All visible 'cells' within each section was labelled in this manner. The XY co-ordinate information was loaded into a routine written by Dr. Paul Hunt in Microsoft Visual Basic 6.0, which calculated the distance in pixels between each individual 'cell' and its closest neighbouring 'cell' based on their XY co-ordinates. The pixel value was then transformed to represent the distance in microns. A table of categories (0-2microns, 2-4 microns etc) upto the maximum recorded distance to closest neighbour was created in Microsoft Excel 2004 for Mac (version 11.6.4) using the output from the Visual Basic routine and each 'cell' was assigned to a category based upon their calculated closest neighbour value in order to determine category frequency. The frequencies were then plotted in a histogram in order to represent cell density as shown by the relative frequency of calculated distances of 'cells' to their nearest neighbour.

Results

Previous work (Ahrens et al. 1977; Wedden et al. 1986) looking at *in vitro* chondrogenesis of limbs and facial processes have used a high density micromass system to induce chondrogenesis in cell suspensions created from explanted embryonic facial tissue. The aim of my work was to transfer this concept to a 3D cell culture system in an attempt to provide a substrate that allows primary cells and tissue to grow in three dimensions as they would *in vivo* as opposed to the two dimensions provided by traditional plasticware.

The chondrogenic system used for this experiment provides a number of interesting areas of investigation. There are two different sources of tissue to be compared, the frontonasal mass and the mandible. They form different structures *in vivo* and when cultured as micromass cultures *in vitro*, allowing comparisons to be made between these two tissues types in 2D and 3D cell culture. Initially these experiments were replicated in 2D before being applied to a 3D cell culture system. There are also two time points to be considered, as the cultures were grown for either 7 or 14 days. The 7 day time point is a continuation of previous micromass work, but the 14 day time point was included to investigate if any differences between cultures were observed when cultured were allowed to grow for a further 7 days. Changes between these two time points will also be compared and contrasted both with each other and between 2D and 3D counterparts.

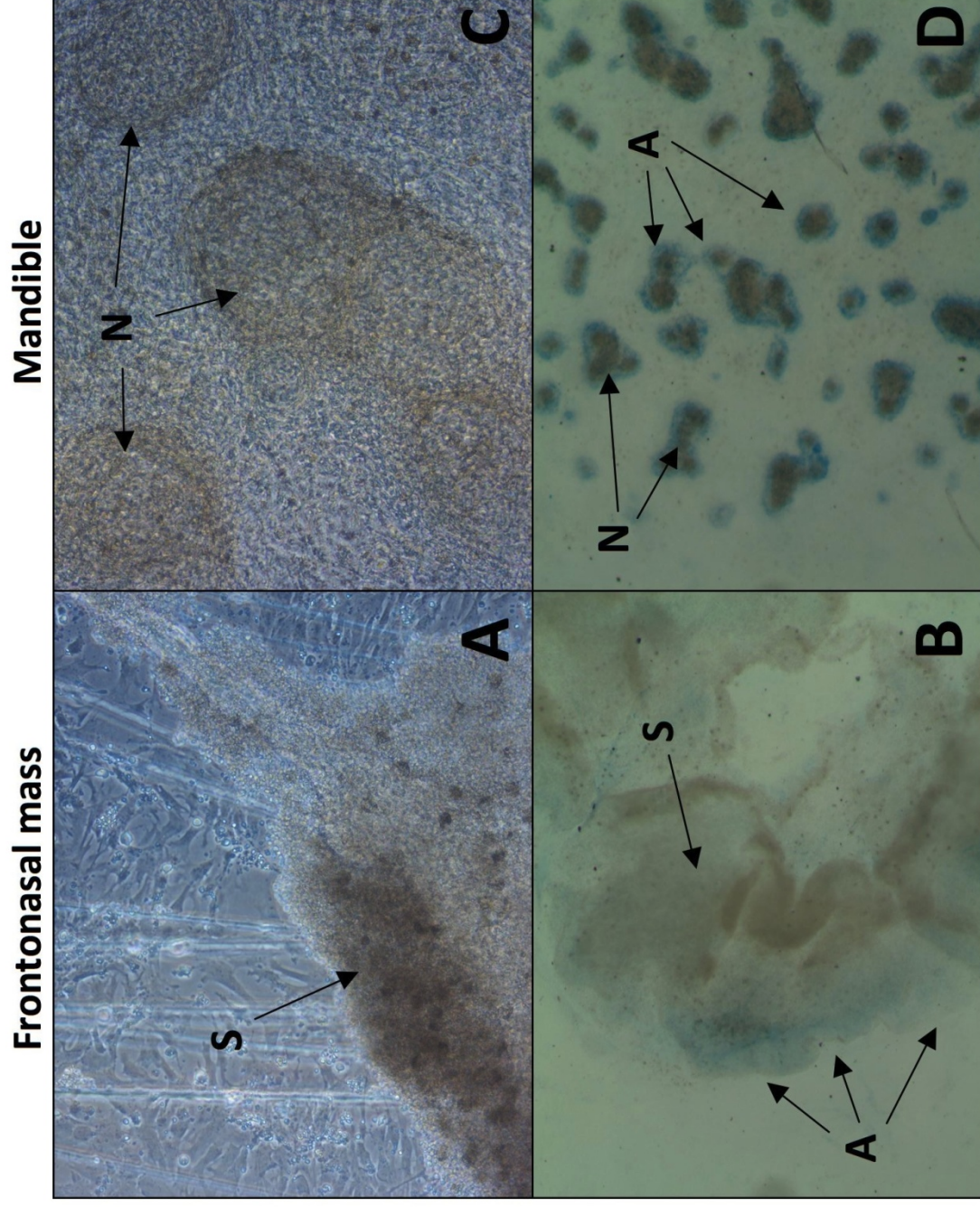


Figure 4: Characteristics of 2D micromass cultures - (A) Phase contrast image of frontonasal mass micromass culture forming a sheet-like population of cells with (B) Alcian blue staining indicating chondrogenic material around the edges of the sheet. (C) Phase contrast image of mandible micromass culture forming nodule structures with (D) Alcian blue staining indicating chondrogenic material at edges of nodules. (Labels: A - Alcian blue staining, N - nodule, S - sheet)

Micromass in a 3D culture system

Micromass cultures of mandible and frontonasal mass cells were created from stage 24 chick embryos and seeded onto traditional 2D plasticware in order to recreate previous micromass experiments before applying the same procedure to a 3D system.

In Figure 4, 2D micromass cultures of mandible and frontonasal mass display different cell behaviours. Cultures of mandibular processes formed nodule-like structures (Fig.4:C,D) while the frontonasal mass cultures form sheet-like structures (Fig.4:A,B) when cultured in 2D, as previously described (Wedden et al. 1986). This differential cell behaviours observed between the two tissue types is likely due to the nature of the different structures the two tissues go on to form *in vivo*, where the frontonasal mass forms a sheet of cartilage that forms the upper beak, and the mandible forms the rod-shaped Meckel's cartilage of the lower jaw. The Alcian blue staining for frontonasal mass cultures shows the stain is restricted to the edges of the sheets (Fig.4:B), and in the mandible cultures, Alcian blue staining is restricted to the edges of the nodules (Fig.4:D). This indicates that chondrogenesis is restricted to the edges of the cultures.

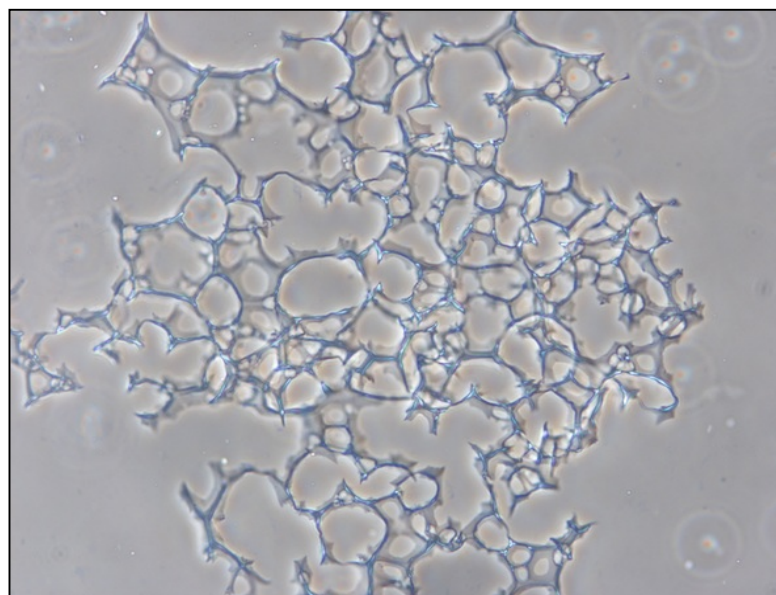


Figure 5: Phase contrast image of alvetex® section – Micromass cultures were unsuccessful in 3D, histological sectioning and staining revealed empty sections of polymer.

3D micromass cultures were not successful. The cell suspensions that were seeded as micromass cultures onto the surface of the polymer discs failed to grow in culture and there was no evidence of the presence of cells within the polymer when discs that had been seeded were sectioned and stained histologically (see Figure 5).

As the protocol used to create this high density micromass system was designed to work on 2D plasticware, changes to the protocol were made to try and adapt the protocol to suit a 3D cell culture system. Cell seeding density was varied for the cell suspension droplet but, again, micromass was unsuccessful. The sterilisation method was changed from plasma treatment to progression through an ethanol series.

In micromass culture the incubation period prior to addition of cell culture medium is required to allow for cell adhesion, addition of the medium too early after inoculation onto the substrate would result in the contents of the droplet being dispersed over the surface of the substrate, thus diluting the high density droplet.

Due to the high wettability of alvetex®, discs tended to absorb the cell suspension droplet as soon as it made contact with the surface of the polymer. This would have dramatically increased the surface area of the droplet. The cell suspension is required to maintain a local area of high cell density in order to induce chondrogenesis within the cell population and so the protocol required modification in order to try and retain a high density droplet on the surface of the polymer.

As the polymer discs require sterilisation and treatment to allow the polymer to be wetted prior to seeding with cells, polymer discs are plasma-treated prior to cell culture. This alters the surface properties of the alvetex® and so the hydrophobicity of polymer is also altered. In order to attempt to decrease the hydrophobic qualities of the alvetex®, an alternative sterilisation treatment progressing discs through an ethanol series through to PBS was applied instead of plasma-treatment.

Discs that had been progressed through an ethanol series were wetted with PBS prior to the addition of the cell suspension droplet and so the droplets were not

so readily absorbed by the polymer scaffold when contact was made with the polymer surface. The droplet was again absorbed although at a slower speed than that observed with plasma treated polymer. The histological stain crystal violet was applied to the incubated polymer discs in order to determine if cells were present on the surface of the polymer. Staining once again revealed no cells were growing on the surface of the polymer (data not shown).

Histological analysis of the polymer discs again showed that the cells had also failed to populate the internal surface of the alvetex® (data not shown). The fact that no cells were present at all in the histological sections indicates that the cells were likely to have dehydrated and died during the 30 minute incubation period prior to flooding with cell culture medium, floated off the surface of the polymer and consequently been removed when cell culture medium was changed.

It is likely that the cell suspension was being absorbed, increasing the surface area occupied by the suspension and dehydrating before a culture could be established on the surface of the polymer. It was concluded that alvetex® was not a suitable substrate for this protocol as it was not possible to maintain a discrete high density cell suspension droplet on the polymer surface. The time taken for cell suspensions to successfully adhere to the substrate exceeds the length of time it takes for cells not submerged in cell medium to dehydrate and die in the incubator.

In order to circumvent this problem a new method of 3D cell culture using whole explanted pieces of mesenchymal tissue was established. By using whole pieces of tissue, the problem of cells spreading too thinly over the surface of the polymer would be avoided as cells would be held together by cell contacts and the extracellular matrix, unlike in the cell suspensions where these contacts were broken to create a single cell suspension. This would avoid the problem of dehydration as the explants could be directly applied to a polymer disc already fully wetted with cell culture medium.

As the prechondrogenic mesenchymal tissue was cultured in intact pieces, this also meant that any microenvironments established while the tissue was undergoing embryogenesis would not be disrupted to the same degree as tissue

that had been broken down into a cell suspension. As a result cultures may retain more *in vivo* – like characteristics than those created from cell suspensions. This may allow for the maintenance of cell types other than early chondroblasts.

This explanted tissue method can also be applied to a 2D system, where explants are simply cultured on the bottom of a cell culture well.

Histological examination of mesenchymal whole tissue explants

In this new protocol, samples of whole pieces of mesenchymal tissue were obtained from the mandible and frontonasal mass and enzymatically stripped of their epithelium. The alvetex® was plasma treated and flooded with cell culture medium prior addition of the explants to allow the polymer discs to become fully wetted with medium before explants were placed on the surface of the polymer. The explants were then cultured for either 7 days or 14 days. 2D counterparts were also cultured where explants were placed on the bottom of a plasma treated cell culture well and also cultured for 7 or 14 days with cell culture medium changes every 48 hours for 2D and 3D cultures.

Cultures were fixed and stained with crystal violet. This staining method allows cells to be located in a culture in order to confirm the presence of cells and examine their growth patterns and morphology. As crystal violet stains cell walls it allows the location of cells without highlighting any other characteristics of the culture other than morphology.

3D cultures were then dehydrated, embedded in paraffin wax, sectioned and histologically stained to reveal their growth patterns within the polymer. It can be difficult to view the actual polymer within histological sections to see whether cell populations are occupying the internal surface or growing on the surface of the polymer. By using phase contrast microscopy and altering the focus to concentrate on the alvetex® substrate rather than the cell populations within the polymer (see Figure 6), polymer in sections can be easily viewed to confirm which part of the polymer is populated.

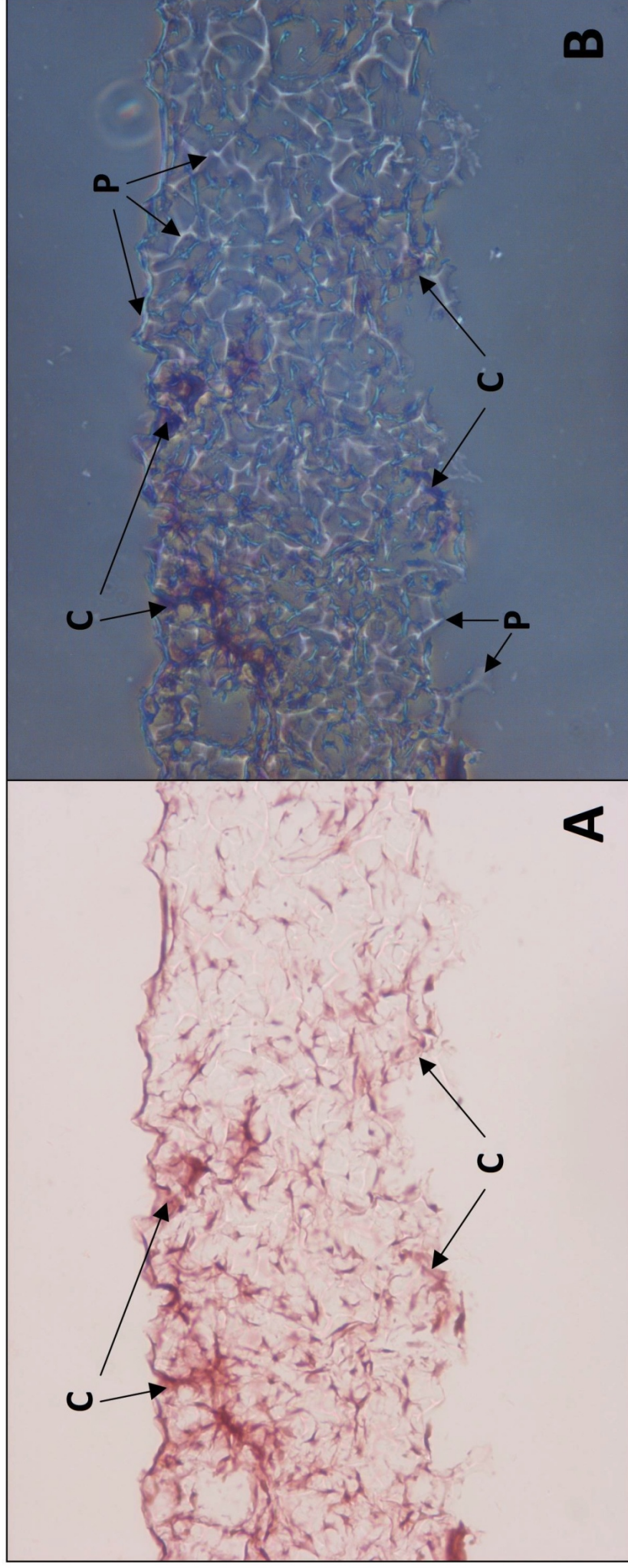


Figure 6: Viewing populated polymer - Using normal inverted light microscopy, while cells growing within the polymer are easy to see (A), the polymer itself cannot be easily viewed. Using phase contrast microscopy (B) on the same samples allows the location of the polymer, which appears as a white matrix with the coloured cells populating the matrix. (Labels: C - cells, P - polymer)

2D growth of mesenchymal explants

Figure 7 shows that whole explants of mesenchymal tissue from frontonasal mass (Fig.7:A) and mandible (Fig.7:B) survive and spread in 2D culture.

Crystal violet staining of explants grown in 2D revealed that the frontonasal mass and mandible had very different cell behaviours.

At 7 days, mandible explant cells have radiated away from the original explants and formed long fibrous structures (Fig.7:D). The dominant structures formed in whole explant cultures are fibrous in nature rather than the nodular structures observed in equivalent micromass cultures. There also appears to be a variation in density in the population with some areas stained more heavily than others.

The 7 day frontonasal mass culture (Fig.7:C) have a similar appearance to micromass cultures where it formed a flat, sheet-like population of cells (Fig.4:A,B) that expanded radially from the original explant. The cell population that extends from the explanted tissue appears to have no distinct fibrous structures visible, however there appears to be some variation in density within the sheet of cells indicated by lighter and heavier patches of crystal violet staining. Low cell density is characteristic of chondrogenic tissue, so areas of lower cell density may be areas of tissue that are undergoing chondrogenesis. It is interesting that while the frontonasal mass grew in a pattern similar to that observed in its equivalent micromass cultures, the mandible appears to have behaved in a different manner and forming a completely different morphology in culture.

These differences after 7 days of culture seem to become less pronounced looking at the 14 day culture samples. The sheet of frontonasal mass cells (Fig.7:E,G) has increased in thickness and appears to be more granular in nature, possibly due to the increased thickness of the cell sheet exaggerating any differences in cell density within the sheet. The edges of the sheet also appear to have peeled away from the plasticware and curled up to leave the original explant somewhat enclosed within a cup-like structure.

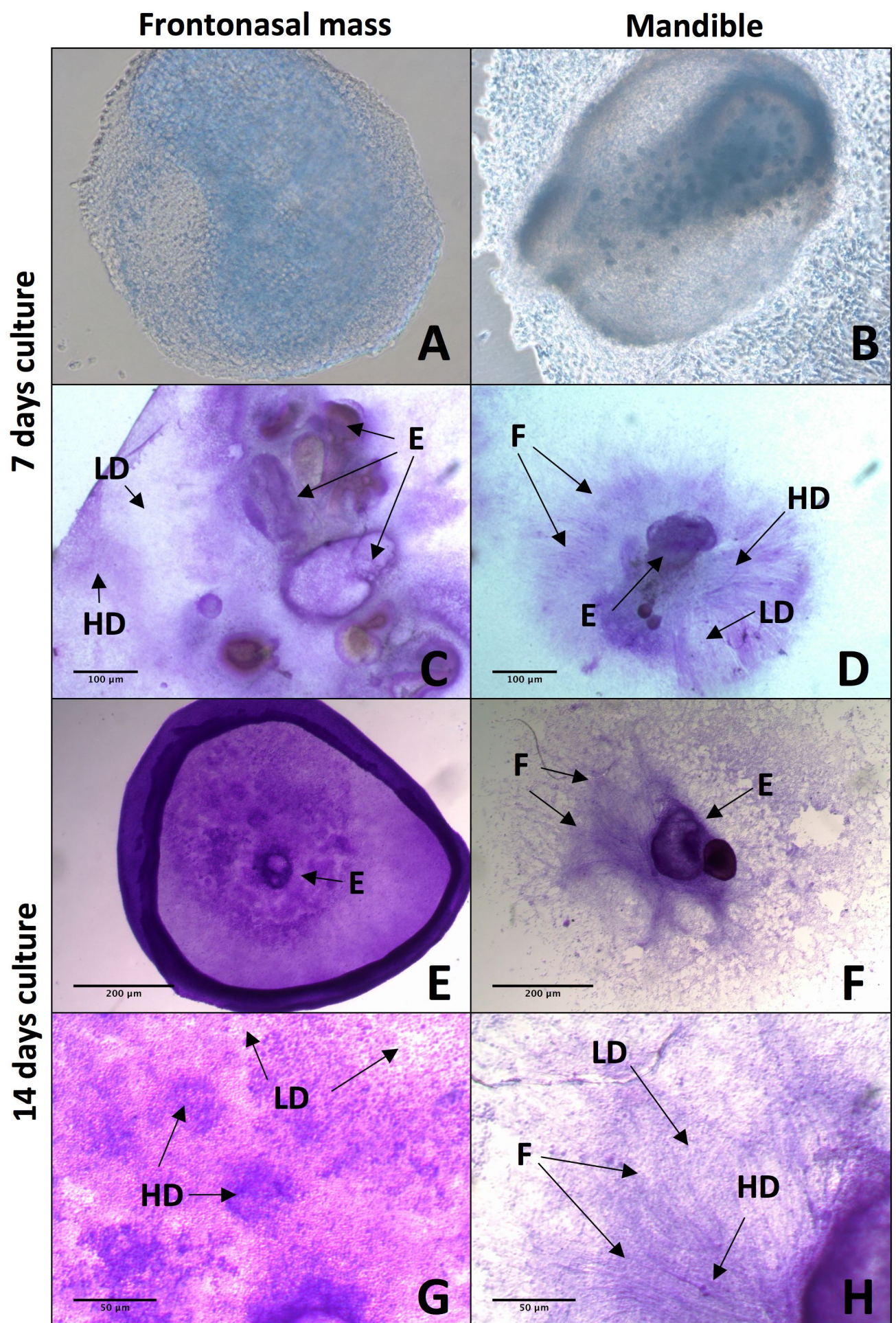


Figure 7: Morphology of 2D explant cultures (A,B) Phase contrast images of explants after 7 days culture (C-H) Crystal violet staining of cultures. (C) Frontonasal mass explants after 7 days of culture, a homogenous sheet-like population of cells extends from the original explants. (D) Mandible explants after 7 days of culture, fibrous extensions radiate from the explant. (E,G) Frontonasal mass after 14 days culture, sheet has thickened with variations in cell density. (F,H) Mandible after 14 days culture, fibrous extensions do not seem to have increased in length, but sheet-like population now surrounds extensions and explant. (Labels: E - explanted tissue, F - fibrous extensions, HD - high density region, LD - low density region)

While thickness of the sheet increased dramatically, it is difficult to tell if the area of the sheet has expanded in a similar manner due to the edges of the culture peeling away from the plasticware.

The 14 day mandibular cultures (Fig.7:F,H) appear to have gained a secondary population of cells that are markedly different from the fibrous structures seen at 7 days. This secondary population of cells are similar to the sheet-like population seen in the frontonasal mass cultures. This probably results from the fact that the original explants contained a different combination of cell types, and that perhaps the mandible contains a fibrous cell type that is unlike the sheet-like cell type seen in frontonasal mass cultures, which grows in a different pattern or at a different rate.

These differences in growth patterns observed between the two tissue types indicates that there are inherent differences between the mesenchyme of the frontonasal mass and mandible, a result that is consistent with previous micromass experiments(Wedden, Lewinsmith et al. 1986). This is not surprising given the fact that these two tissue sources form completely different cartilage structures, both *in vivo* and in micromass cultures.

Attempts were made to embed these explants in paraffin wax, section and stain histologically, but due to the delicate nature of the structures formed by the explants, they did not maintain their form through the procedures.

3D growth of frontonasal mass mesenchymal explants at 7 days

Figure 8 shows the growth of a frontonasal mass explant after 7 days of culture in alvetex®. The original explant is clearly visible on the surface of the polymer (Fig.8:A), both on the histological section and in a whole mount crystal violet staining of explants (Fig.8:C).

Cells have populated the inner surface of the polymer. The explant itself appears to have three distinct cell layers (see Figure 9), an outer layer of flattened fibroblastic-like cells, and inner population of cells with a more rounded phenotype with a low cell density layer separating the two.

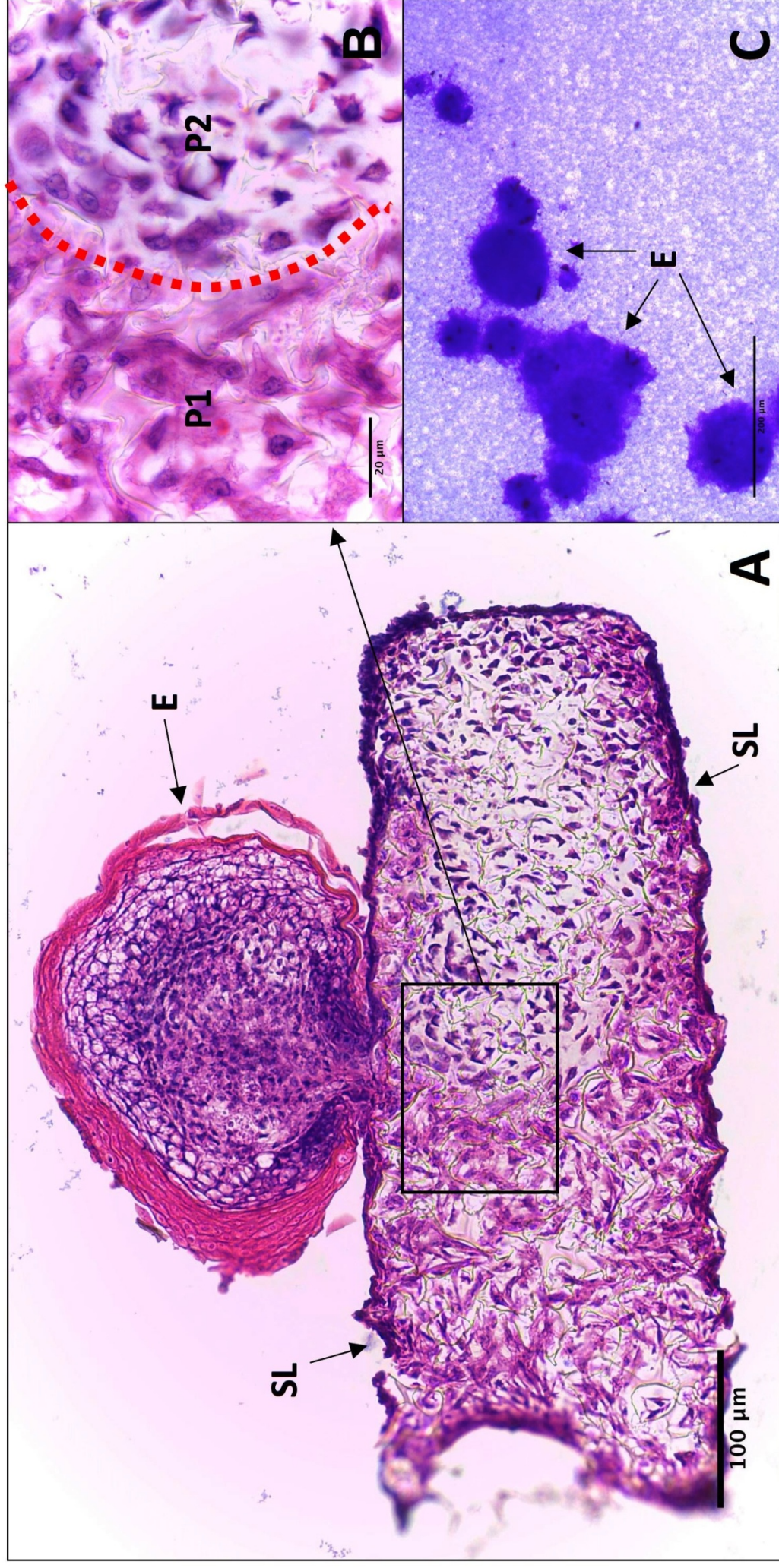


Figure 8: Frontonasal mass explants grown in 3D culture for 7 days - (A) H&E staining of 10µm section - frontonasal mass explant on the surface of the alvetex®. Cells appear to have migrated into and populated the polymer substrate. There are layers approx 2-4 cells deep on the top and bottom layers of the polymer. There appears to be two distinct populations of different cell density as indicated by (B) with red dotted line indicating interface between P1 the high density population and P2 the low density population. (C) Whole mount in-situ crystal violet stained frontonasal mass explants on the surface of the polymer disc. (Labels: E - explant tissue, SL - polymer surface cell layer, P1 - cell population 1, P2 - cell population 2)

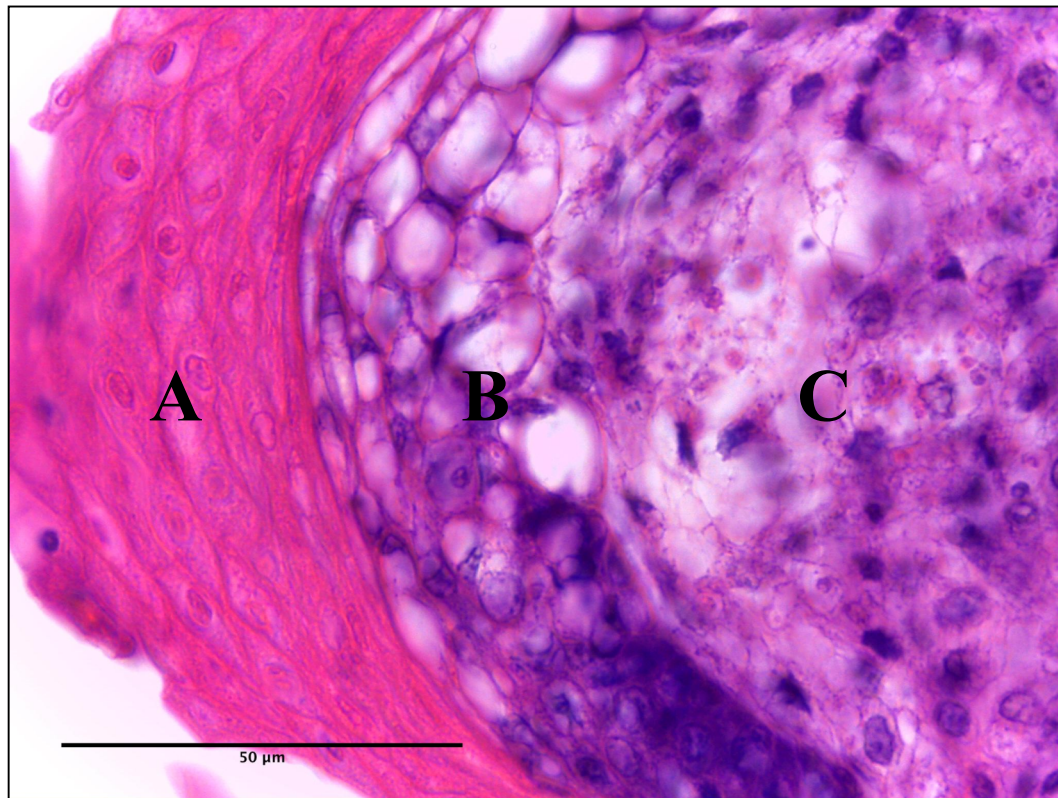


Figure 9: Frontonasal mass explant at 7 days – There are three distinct layers present (A) the outer flattened layer, (B) low cell density layer and (C) inner population of rounded cells

There is a disruption in the outer layer of the explant that connects to the surface of the polymer, giving the impression that cells are ‘emptying’ from the inner cell population of the explant into the polymer substrate and then populating it. On the basis of their similar morphologies I believe it is the cells from the inner cell population of the explant that are populating the polymer rather than cells from the other layers of the explant. The cells are occupying the entire depth of the polymer and have formed a layer of cells on the top and bottom surfaces that extend beyond the polymer to form a layer 2-4 cells in depth (Fig.8:A).

Within the actual polymer itself there appears to be two different cell populations, one with a higher cell density than the other (Fig.8:B). The population with the lower cell density is reminiscent of chondrogenic tissue, with the low cell density and little cytoplasm, whereas the other cell population appears to be more fibroblastic in nature with a higher cell density. In chondrogenic tissue *in vivo*, the chondrogenic material is an area of low cell density surrounded by a periphery population of high density cells that allow the

chondrogenic population to maintain its low cell density after cell condensation has occurred. The area of low cell density present in these cultures may be an area that is undergoing chondrogenesis while the other cell population may be a periphery population that allows the cells to undergo chondrogenesis.

The crystal violet staining shows the location of the explants in the polymer disc (Fig.8:C). Taking the more heavily stained portions of the population visible as being the original explants, the cells do not appear to have migrated very far from the explants and there is little evidence of their presence within the polymer beyond the area immediately surrounding the explants. This seems to be consistent with the histological sections, which only have cells populating the polymer approximately 200um from the original explant (Fig.8:A). The population expansion from the explant has also included downwards migration into the scaffold, as well as the lateral radial migration along the polymer surface. Due to the opacity of the polymer it is difficult to visualise live cells once they have migrated down into polymer, however, a fluorescent live cell tracking system has been developed to attempt to overcome this problem (see *Live cell tracking of Dil labelled mesenchymal tissue explants*).

3D growth of frontonasal mass mesenchymal explants at 14 days

Figure 10 shows the extent to which the cells have populated the polymer from a frontonasal mass explant after 14 days of cell culture. Population of the alvetex® by the explant has extended far beyond that seen in the 7 day explants. Figure 8 (image A) shows that the extent of growth from the original explant is approximately 200um, whereas the extent of growth at 14 days (Fig.10:A) exceeds 1500um from the site of the original explant.

There no longer appears to be the same two distinct populations of cells that are observed in the 7 days explant cultures. Although there is an area of higher cell density towards the centre of the section, this is likely where the 'entry' point for cells emptying from the explant into the scaffold was located.

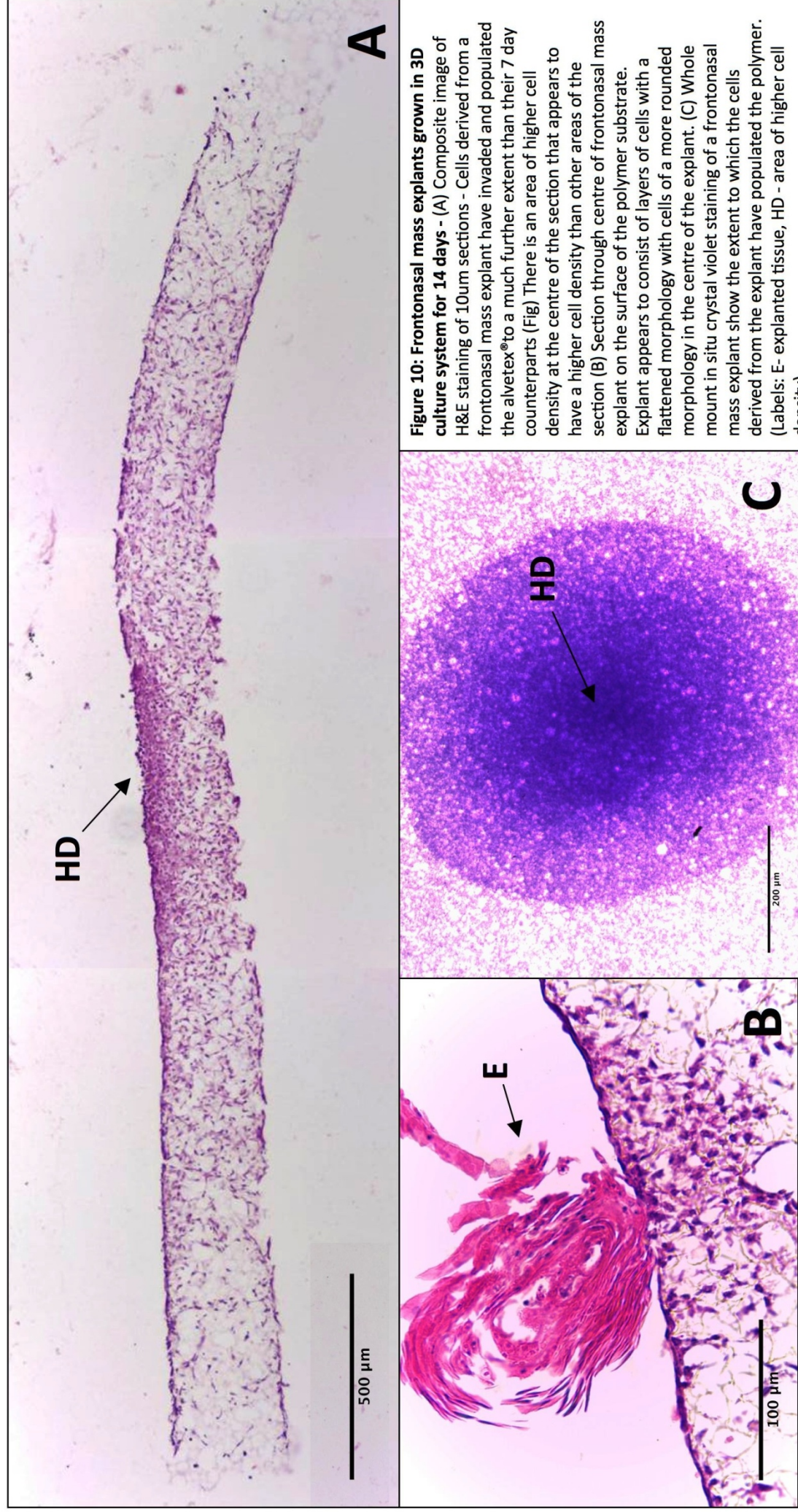


Figure 10: Frontonasal mass explants grown in 3D culture system for 14 days - (A) Composite image of H&E staining of 10µm sections - Cells derived from a frontonasal mass explant have invaded and populated the alvetex® to a much further extent than their 7 day counterparts (Fig) There is an area of higher cell density at the centre of the section that appears to have a higher cell density than other areas of the section (B) Section through centre of frontonasal mass explant on the surface of the polymer substrate. Explant appears to consist of layers of cells with a flattened morphology with cells of a more rounded morphology in the centre of the explant. (C) Whole mount in situ crystal violet staining of a frontonasal mass explant show the extent to which the cells derived from the explant have populated the polymer. (Labels: E- explanted tissue, HD - area of higher cell density)

As evidence so far suggests that the population expands in a lateral radial manner from the original central explant, this central area of locally high cell density can logically be assumed to be the site at which the original piece of explanted material was located. As the crystal violet whole mount staining shows (Fig.10:C), there is a radial expansion of the cell population from the centre of the culture where the explant would have been located. The area of the polymer occupied by the lateral radial expansion can again be seen to be much greater than that seen in its 7 day equivalent and has extended beyond just revealing the location of the explanted material as was shown at 7 days (compare Fig.10:C to Fig.8:C).

Aside from the central region, when comparing the 14 day and 7 day cultures, the majority of the cells in the 14 day culture seem to be at a lower density than the culture at 7 days (compare Fig.10:A to Fig.8:A). There is a region of high cell density, but it seems to be restricted to a small central region surrounded by an area of lower cell density. As before it was speculated with the 7 day cultures that the area of lower density was chondrogenic tissue, the fact that this area of low density seems to have expanded to become the majority of the cell population may be indicative in an increase in the amount of chondrogenic tissue present.

In some cases the remaining cells from an original piece of explanted tissue can also be seen (Fig.10:B). When compared to the 7 day explant on the surface of the polymer, it appears to have lost the majority of the inner cell population and only the flattened cells of the outer layer remain with a few more rounded cells remaining in the centre of the explant. The number of flattened layers appear to have increased between 7 and 14 days, and is observed in multiple serial sections of the explant at 14 days. The cells that remain in the explanted material on the surface of the polymer do not have a similar morphology to the cells that have populated the internal surface of the polymer, giving further evidence that the cells from the inner layer of the original explant are the cells that have migrated into and populated the polymer, leaving behind the cells from the other layers of the explants.

3D growth of mandible mesenchymal explants at 7 days

Figure 11 shows mandible explants that have been cultured on alvetex® for 7 days. As with the frontonasal mass explant (Fig.8) the original explant is clearly visible on the surface of the polymer, however its appearance is very different to that of the frontonasal mass explant. The mandible explant only appears to have two different cell layers, a dense outer layer of cells and an inner cell mass of significantly lower cell density (Fig.11:A). As with the frontonasal mass there seems to be a point of 'entry' where cells are emptying from the inner cell mass into the polymer substrate below and within the polymer itself, again there appears to be distinct populations of cells, a population of low cell density and a population of high cell density (Fig.11:B). By looking at the areas of polymer that are directly in contact with the explant (Fig.11:A), while there is a point of entry for the cells that are invading from the inner mass of the explant, the cells that make up the population of high cell density appear to originate from the outer layer of the explant and so there are two distinct populations of cells within the inner surface of the polymer, as opposed to the frontonasal mass which only appears to populate the polymer with cells from the inner population of cells from the explant (Fig.8:A).

As with the frontonasal mass, the cells are occupying the entire depth of the polymer substrate with layers beyond the polymer that are 2-4 cells in depth on the upper and lower surfaces of the disc (Fig.11:A).

The explant is clearly visible with whole mount in situ crystal violet staining of the polymer disc (Fig.11:C) and a small area of migration can be seen surrounding the explanted tissue. Unlike its 2D counterpart there does not appear to be any obvious evidence of the fibrous outgrowth from the explants, a distinctive characteristic of the 2D cultures at 7 days.

As with the frontonasal mass, the extent population outgrowth from the explanted tissue increases from 7 day cultures to 14 day cultures. At 7 days, the growth of the population from the explant shown in Figure H reaches to an extent of approximately 400um (data not shown), but by 14 days the extent of the population has expanded to approximately 1000um from the site of the original explant (Fig.12).

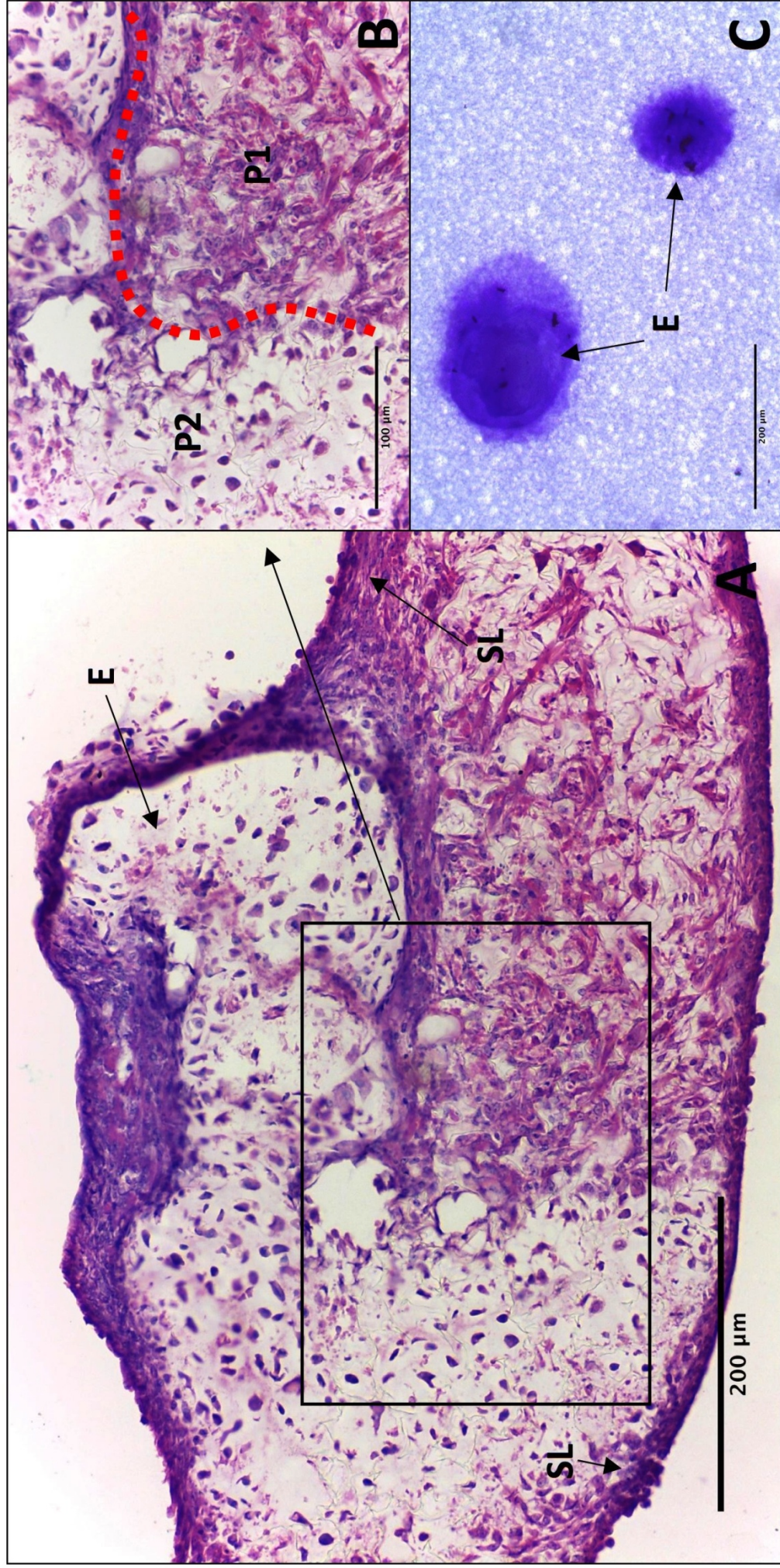


Figure 11: Mandible explants grown in 3D culture for 7 days - (A) H&E staining of 10μm section - Mandible explant on the surface of the alvetex®. There appears to be layers approx. 2-4 cells thick on the top and bottom surfaces of the polymer. As with the frontonasal mass explant (Fig.), cells from within the explant appear to have migrated into and populated the polymer substrate. There are also appears to be areas of differing cell density (B) as seen in the frontonasal mass samples, with P2 appearing to have a lower cell density than P1. Red dotted line indicates interface between two cell populations. (C) Whole mount in-situ crystal violet stained mandible explants on the surface of the polymer disc. (Labels: E - explanted tissue, SL - polymer surface cell layer, P1- cell population 1, P2 - cell population 2)

This indicates that the mandible culture population may expand to occupy more of the polymer at 7 days than the frontonasal mass culture equivalent, but by 14 days, the extent of the frontonasal mass population seems to have exceeded that of the equivalent mandible cultures. This observation that the extent of population of the polymer in frontonasal mass exceeds that of equivalent mandible cultures was observed in all cases and across multiple culture samples (data not shown).

3D growth of mandible mesenchymal explants at 14 days

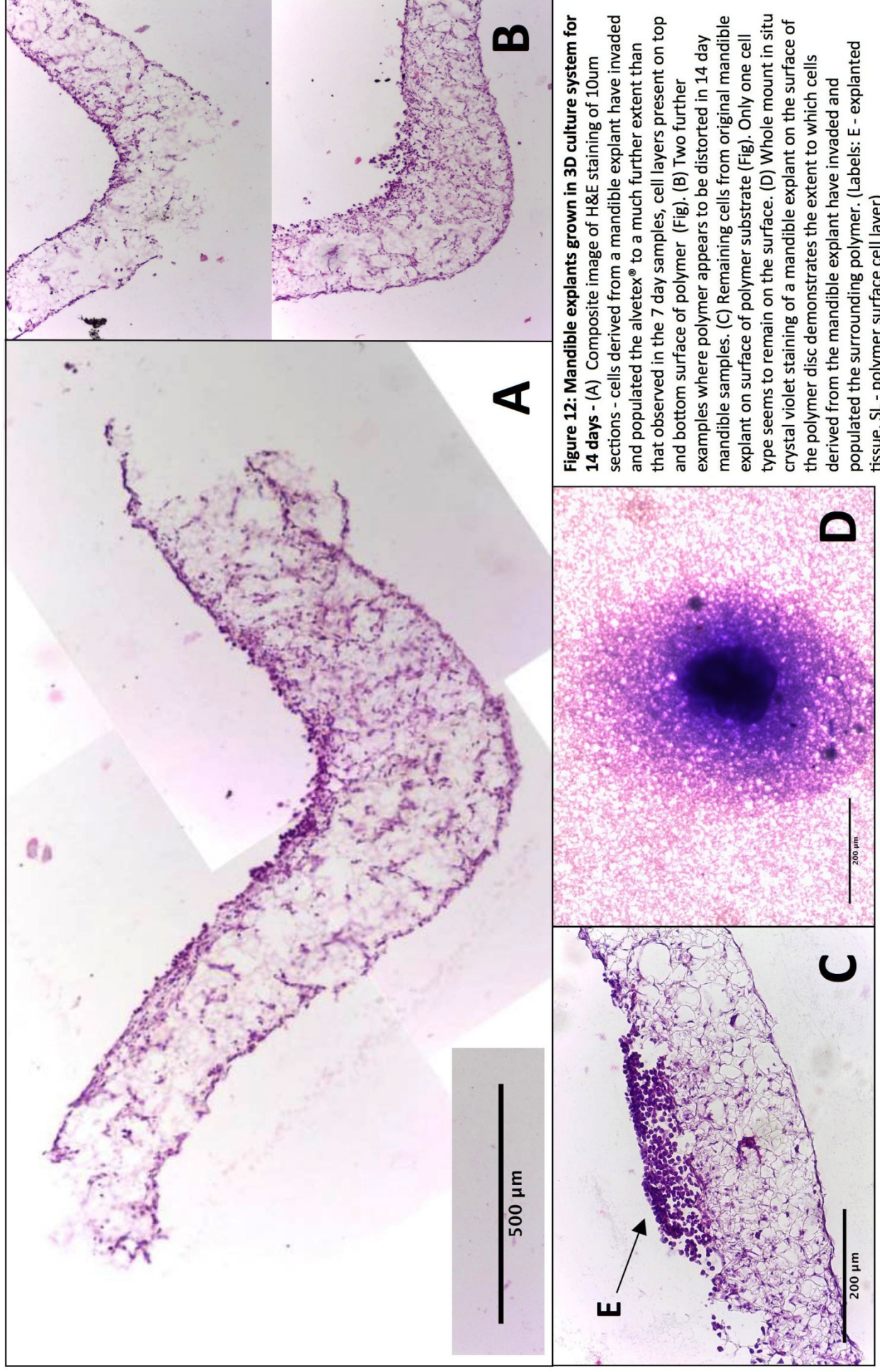
After 14 days of culture in a 3D system (see Figure 12), the appearance of the mandible culture is significantly different to its 7 day counterpart.

Again as seen with the frontonasal mass 14 day cultures, there are no longer two distinct populations of cells within the polymer itself. The cells have spread in a radial lateral manner from the explant to further populate the available space in the polymer substrate (Fig.12:A).

The cell density appears to be lower than that observed in both its 7 day counterpart and that observed in 14 day cultures of frontonasal mass. If this is in fact indicative of chondrogenic tissue, then it would appear that the mandible culture produces a lot of chondrogenic tissue.

Multiple layers of cells that may represent the remains of an explant can be seen on the surface of the polymer (Fig.12:C). As with the frontonasal mass, cells that appear to be derived from the outer layer of the explant remain on the surface while cells from the inner mass have migrated into the alvetex®, although the similarity in remaining explant cell layers (Fig.12:C) and explant cell layers observed at 7 days (Fig.11:B) is not as pronounced as the equivalents in frontonasal mass cultures.

What is striking about the mandible cultures at 14 days is the 'U' shape of the histological sections (Fig.12:B). This distortion of the polymer appears consistently in sectioned mandible cultures at 14 days. One possibility is that a population of myoblastic cells are populating the top layer of the polymer and contracting, causing the polymer to distort into this 'U' shape. The upper surface



of the polymer appears to have a thicker layer of cells, which could be this myoblastic population (Figure 13).

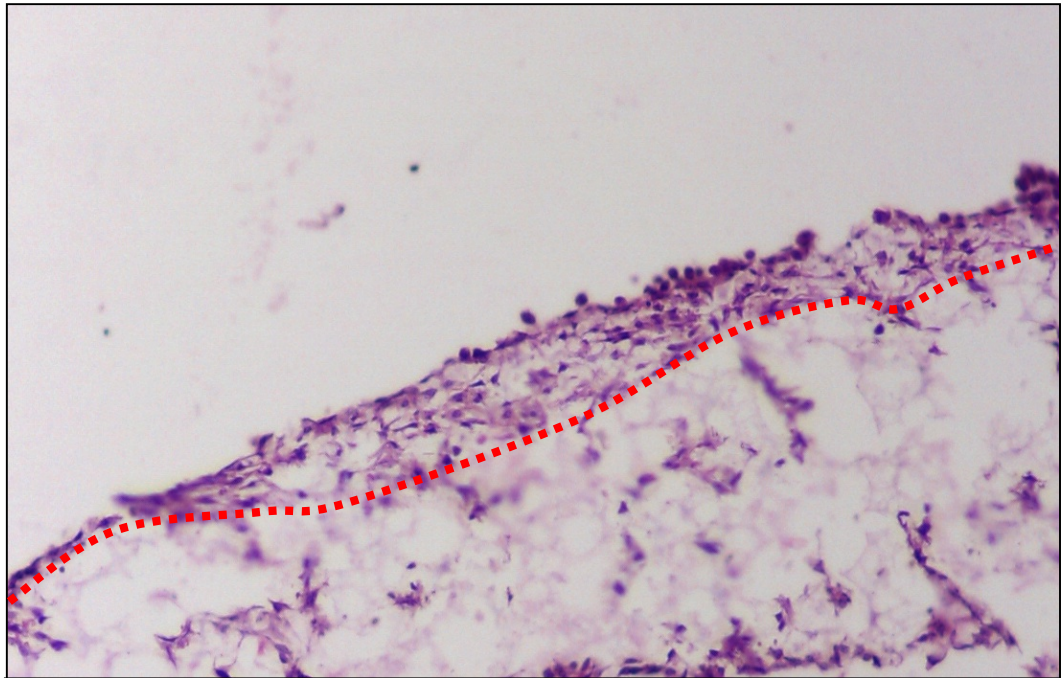


Figure 13: Upper surface of 14 day mandible culture – Layer of cells on the upper surface of the polymer (red dotted line indicates top of polymer) could be a myoblastic population whose contraction causes the polymer to distort into a 'U' shape

Comparing cell densities between tissue types and time points in 3D

As described in the histological examination of explanted mesenchymal tissue, the cell densities of the two tissue types appeared to vary both from each other and the two time points of 7 days and 14 days of 3D cell culture.

To quantify this, the relative cell densities of the two tissue types were calculated for the two different time points and is represented by determining the distance between a cell and its closest neighbouring cell. For areas of low cell density, the distance between a cell and its nearest neighbouring cell will be much greater than that of areas of high cell density and so histograms representing distances between neighbouring cells within a section of populated polymer can give a representation of cell density.

Cell density at 7 days

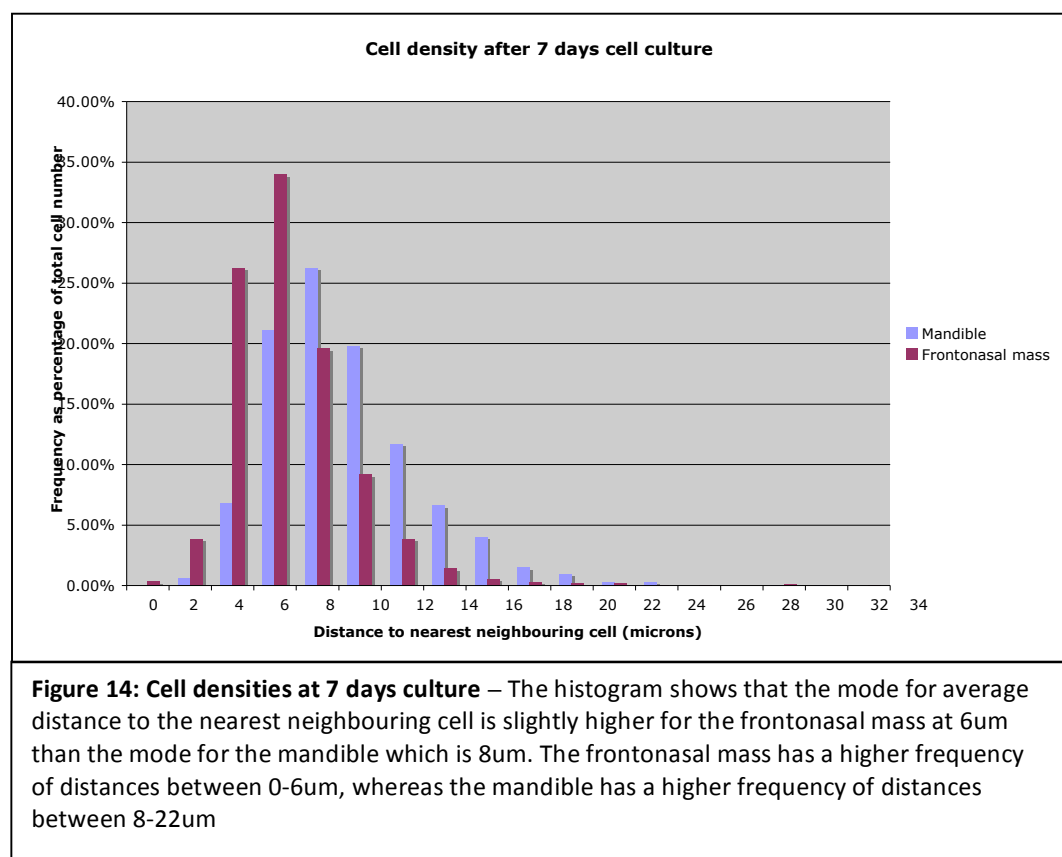


Figure 14 indicates that the mode for the frontonasal mass is higher than that of the mandible, where the mode for the frontonasal mass is 6um as compared to the 8um of the mandible. This appears to be only a very small difference in cell

density, but looking at the frequency at which these densities occur shows that the frontonasal mass has a much higher frequency of distance to nearest neighbour between 0 and 6µm than the mandible, which has higher frequencies of 8 to 22µm than the frontonasal mass.

This indicates that while the mode for the two tissue types may be very similar, the frequency of the occurrence of distance that indicates a high cell density (i.e. 0-6µm) is much higher in the frontonasal mass than the mandible, thus implying that the frontonasal mass has a higher frequency of areas of high cell density. Conversely the mandible has higher frequencies of distances that indicate a low cell density (8-22µm), implying that the mandible has a higher frequency of areas of low cell density. This is consistent with the histological evidence that the mandible has larger areas of low cell density than the frontonasal mass and may be indicative of larger areas of chondrogenic tissue.

Cell density at 14 days

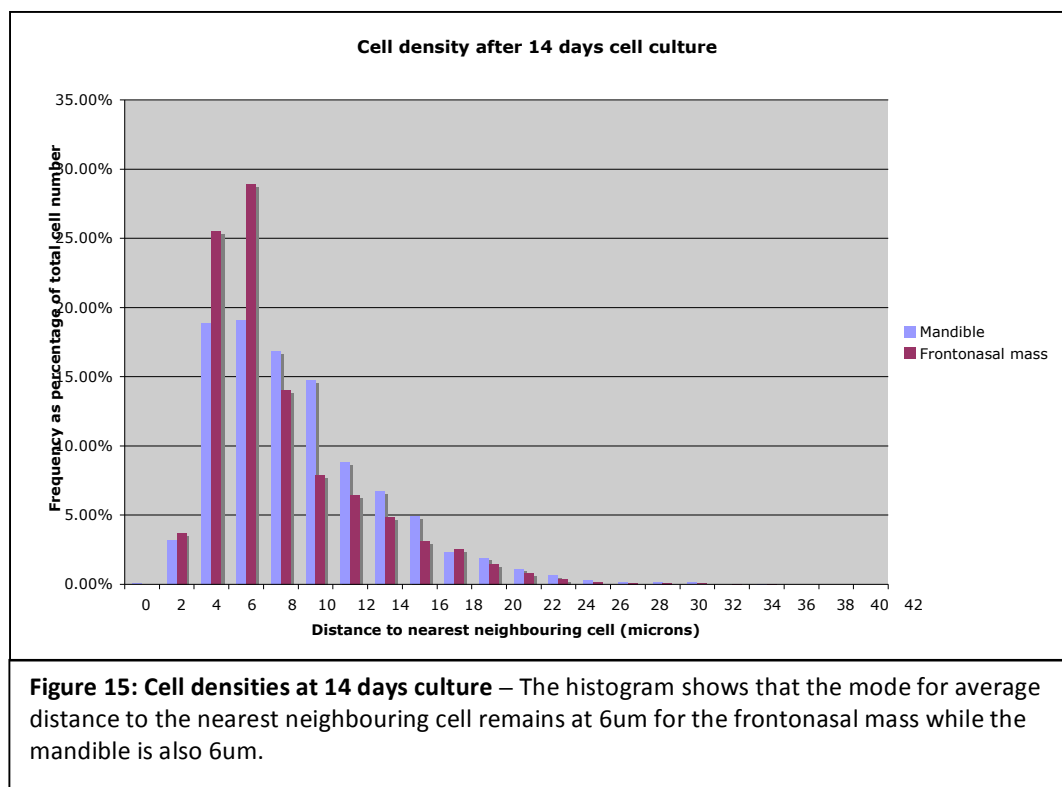


Figure 15 shows that at 14 days the mode for the frontonasal mass has remained at 6µm, but the mode for the mandible has decreased to 6µm, representing an increase in actual cell density. This result seems to be at odds

with the histology, which indicates that the mandible has a lower cell density than the frontonasal mass. A possible explanation for this discrepancy is the presence of the layer of higher density cells on the top surface of the mandible explant culture (Fig.13). These cells have a higher cell density than the cell population of the internal surface of the polymer and so when this cell layer was included in the cell counts, they may have skewed the results to appear as if mandible explant cultures had a higher modal cell density within the polymer than is actually the case. Figure 16 shows that when the high density population on the top surface of mandible explant cultures are excluded from cell counts to determine cell density, the mode returns to the 8um that was previously observed in 7 day cultures.

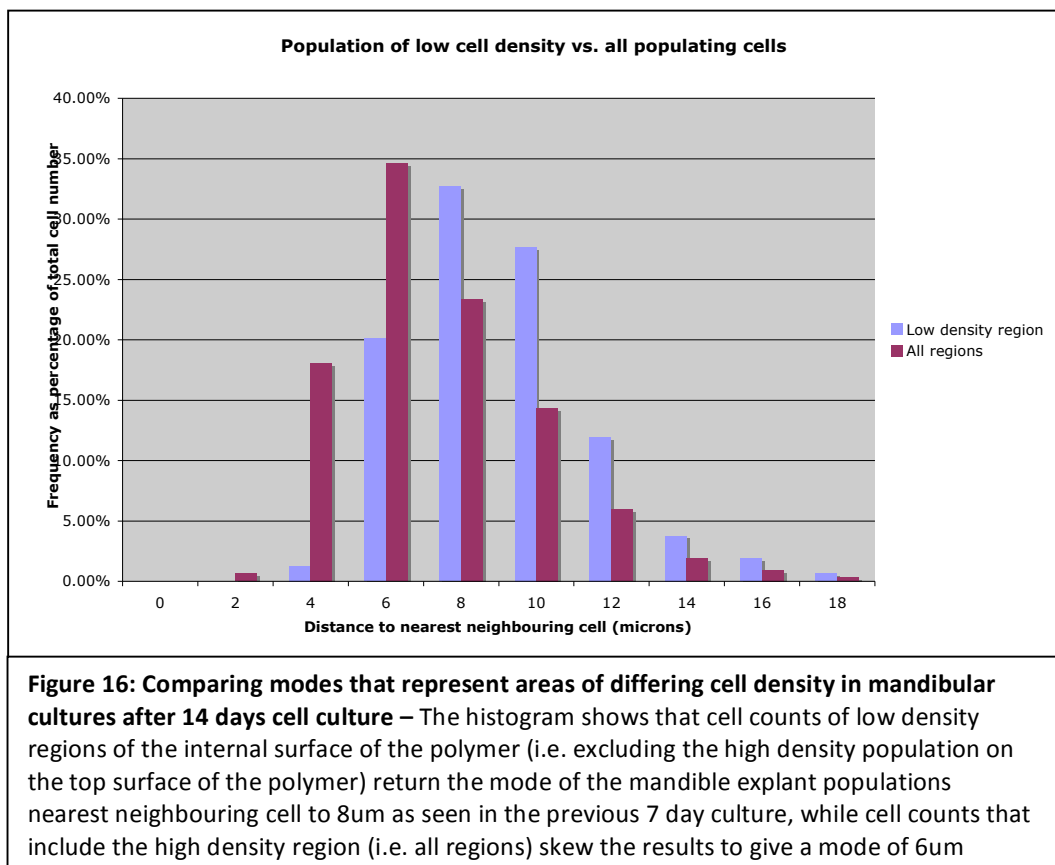
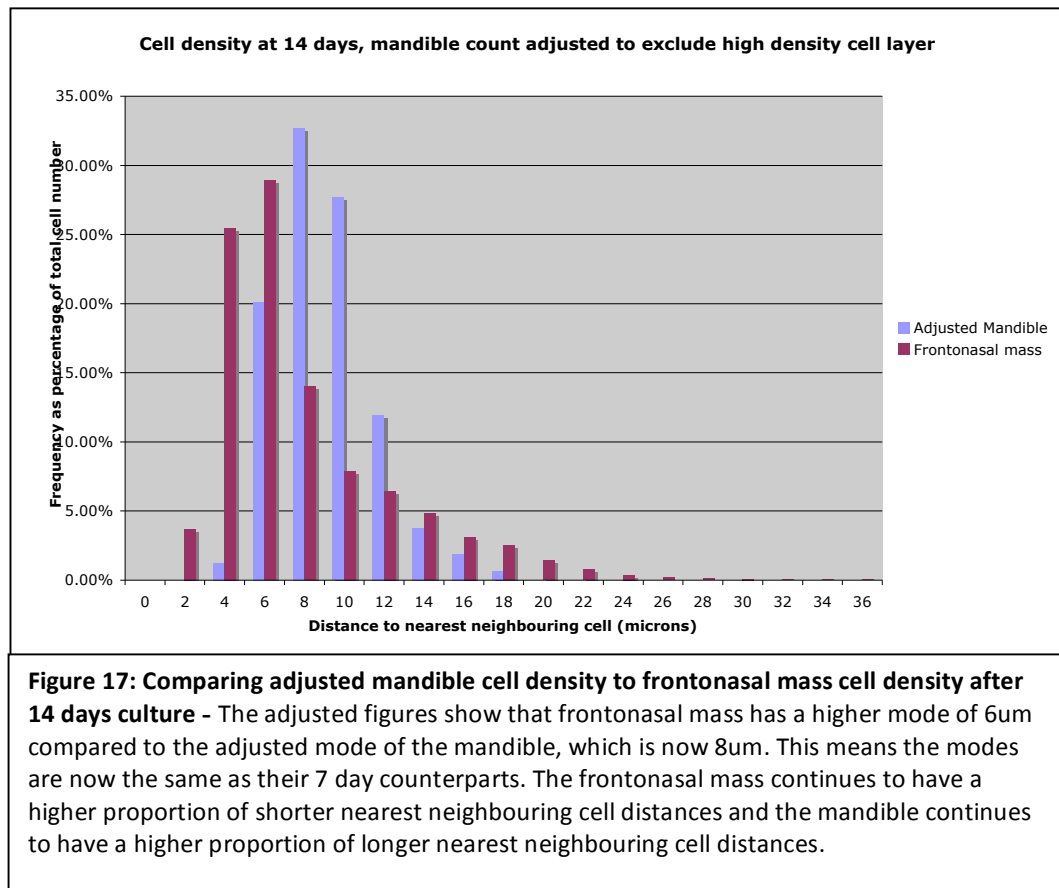


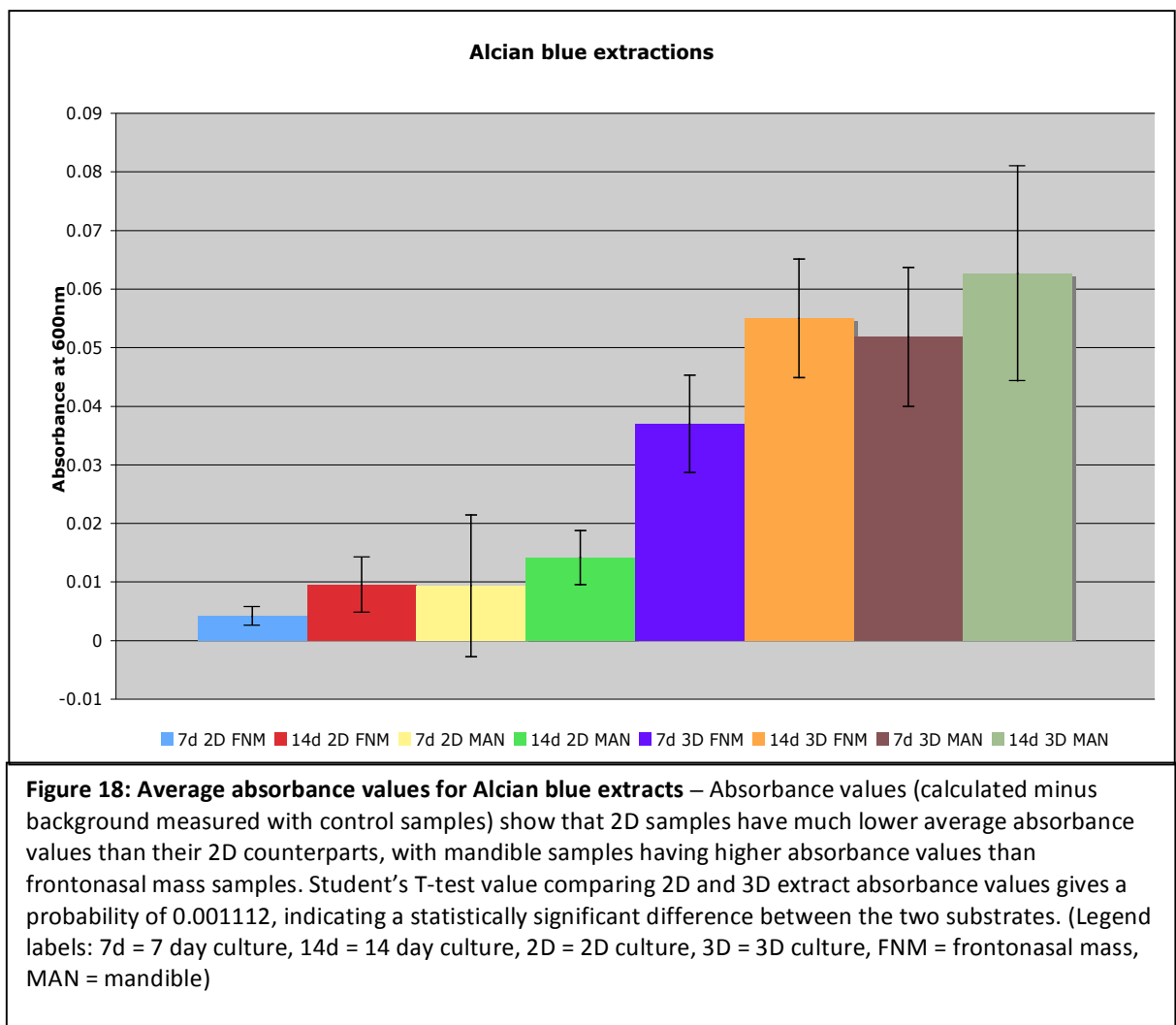
Figure 17 shows that when these adjusted mandible density counts are plotted against the frontonasal mass density counts, it shows that the modes are now both the same as their 7 day equivalents, and that the frontonasal mass retains a higher frequency of high cell density, and the mandible retains a higher frequency of low cell density.



This evidence shows that for both frontonasal mass and mandible cultures, there is no change in cell density between 7 and 14 days culture, it remains constant as the cell populations expand and further populate the polymer. Cell density seems to be an intrinsic property of these cell populations and is dependant upon the tissue of origin. The 3D environment has maintained this intrinsic difference in culture.

Alcian blue staining and the presence of chondrogenic tissue

To attempt to quantify the differences in levels of chondrogenesis in different culture regimes, I used Alcian blue staining. The histological stain Alcian blue indicates the presence of sulphanated glycosaminoglycans, a major component of cartilaginous matrix present in chondrogenic tissue. Alcian blue stain was applied to both 2D and 3D cultures of the two types of explanted tissue after 7 day and 14 day culture periods. The amount of stain taken up by the cultures should give an indication of the amount of cartilaginous tissue present in each of the cultures, so in order to determine the amount of stain taken up by each cultures the stain was extracted and its absorbance measured at 600nm. The higher the absorbance value, the more stain is present in the extract and a therefore a higher proportion of cartilaginous matrix is present in the original culture.



The absorbance values in Figure 18 show that the 3D samples have a higher average absorbance value than their 2D counterparts indicating a higher proportion of chondrogenic tissue present in the 3D samples. Control samples of alvetex® were fixed and stained with Alcian blue and run through the same extraction protocol as alvetex® with explanted tissue cultured on them which revealed no difference between the background control and the stained empty discs, so any difference in absorbance cannot be attributed to stain being absorbed and retained by the polymer. This provides evidence that the alvetex® has a more conducive environment for the production of chondrogenic material from explanted mesenchymal tissue than explants that are grown on traditional 2D plasticware.

	Tissue origin	2D average	3D average	Fold-increase in 3D
7 days	Frontonasal mass	0.004167	0.037	8.88
	Mandible	0.009333	0.0518333	5.55
14 days	Frontonasal mass	0.0095	0.055	5.79
	Mandible	0.014167	0.062667	4.42

Table 1: Calculating the increase in Alcian blue absorbance between 2D and 3D samples –
Highest increase of 8.88-fold occurs between 7 day frontonasal mass samples, with the smallest increase being 14 day mandible samples at 4.42-fold

Table 1 shows that the frontonasal mass 7 day cultures experienced the highest increase in Alcian absorbance with an 8.88-fold increase in absorbance and the mandible explant 14 day cultures had the smallest increase with a 4.42-fold increase. These figures help to illustrate the dramatic increase in the presence of Alcian positive material in mesenchymal tissue grown in a 3D cell culture system compared to their 2D counterparts grown on traditional plasticware.

As it was observed in crystal violet staining of the 2D cultures, there may have been some differences in cell density in the cultures that may be due to the formation of areas of chondrogenic tissue, which would have an overall lower cell density. The absorbance values above indicate that there may have been some chondrogenesis occurring as there is a small amount of Alcian blue stain

that can be extracted from the samples, more so from the mandible samples, but nowhere near the amount that was extracted from 3D samples.

In all cases, mandible explant cultures have a higher absorbance value than frontonasal mass cultures. This is concurrent with histological evidence presented above which appears to demonstrate an increased number of areas of low cell density (compare Fig.8:A & Fig.10:A to Fig.11:A & Fig.12:A) consistent with the chondrogenic tissue phenotype. The fact that the Alcian blue absorbance is high with these samples provides further evidence that mandibular samples have a higher proportion of chondrogenic material than frontonasal mass tissue explant cultures.

Further evidence regarding the presence of chondrogenic tissue is provided when sections of the cultures are stained with Masson's Trichrome, which stains collagen containing tissue blue. Figure 19 shows that areas that have been identified as of low cell density and speculated to be chondrogenic tissue have been stained blue by Masson's Trichrome, indicating that collagen may be present. As collagen is a major component of cartilaginous extracellular matrix, this is another indication that the areas of low cell density are chondrogenic tissue.

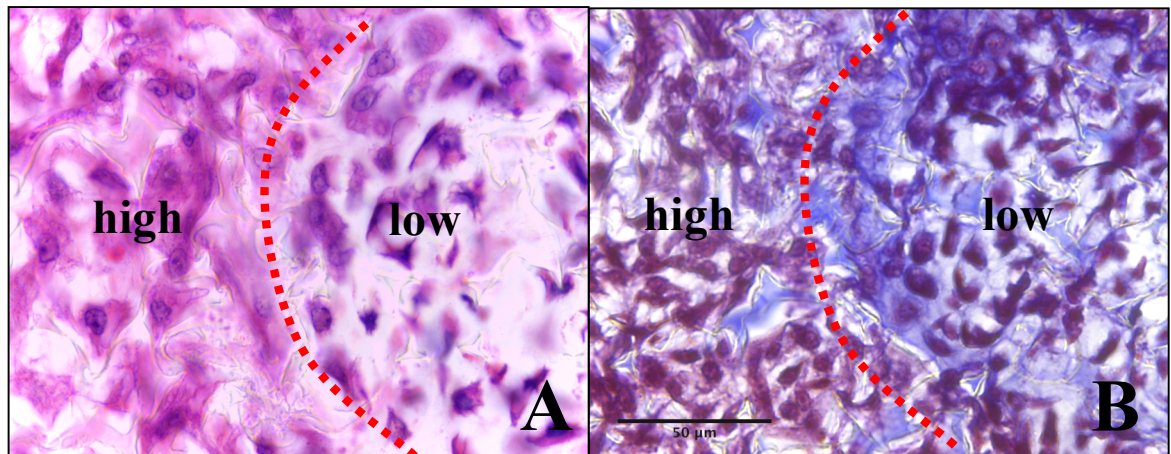


Figure 19: Indication of presence of collagen in areas of low cell density – (A) H&E staining showing region of low cell density on right of dotted line (B) Masson's Trichrome histological stain shows that the areas of low cell density that were speculated to be regions of chondrogenic tissue are stained blue, and indicator of the presence of collagen, a major component of cartilaginous extracellular matrix (high – high cell density, low – low cell density)

Live cell tracking of Dil labelled mesenchymal tissue explants

As the 3D cell culture system used in this series of experiments is opaque, any cells that occupy the internal surface of the alvetex® cannot be visualised until the disc has been fixed and sectioned, and so live pictures of cells within the polymer cannot be obtained, making it difficult to continuously monitor the behaviour of living cells in culture. For long term cultures this can mean that it is not possible to establish if cells have grown in alvetex® except for histological analysis at the end of culture period. Up until this time, populated and empty polymer discs can look identical. By labelling the explants with Dil, cells within the polymer can be located within the polymer and give an indication as to the progress of the cell population within the polymer, a tool that has been pioneered in this experimental work.

Fluorescent labelling of explants with Dil prior to culture allows the migration of cells to be observed in live cultures. This means that the progressive outgrowth of cells from a single explant can be monitored and recorded and so an idea of the pattern of growth and migration can be obtained.

Tracking frontonasal mass tissue explants in 2D

Dil labelled pieces of tissue were visible under an inverted fluorescence microscope at Day 1 and so cell populations could be tracked. Although it was difficult to identify individual cells, by comparing images taken every 48 hours, it was possible to show the cells migrating in a lateral radial fashion from the original explant (Figure 20), as had been seen in the 2D crystal violet stained examples.

As the cells migrated further from the original explant and the cell population expanded, the intensity of the fluorescent dye was diluted as can be seen with images taken from Day 5 (Fig.20:C,G) and 7 (Fig.20:D,H), at Day 9 it was no longer possible to locate the cell populations (data not shown).

Tracking mandible tissue explants in 2D

Figure 20 shows the migration of cells derived from mandibular tissue in a 2D environment. Unlike the frontonasal mass (Fig.20:A-D), which appears to

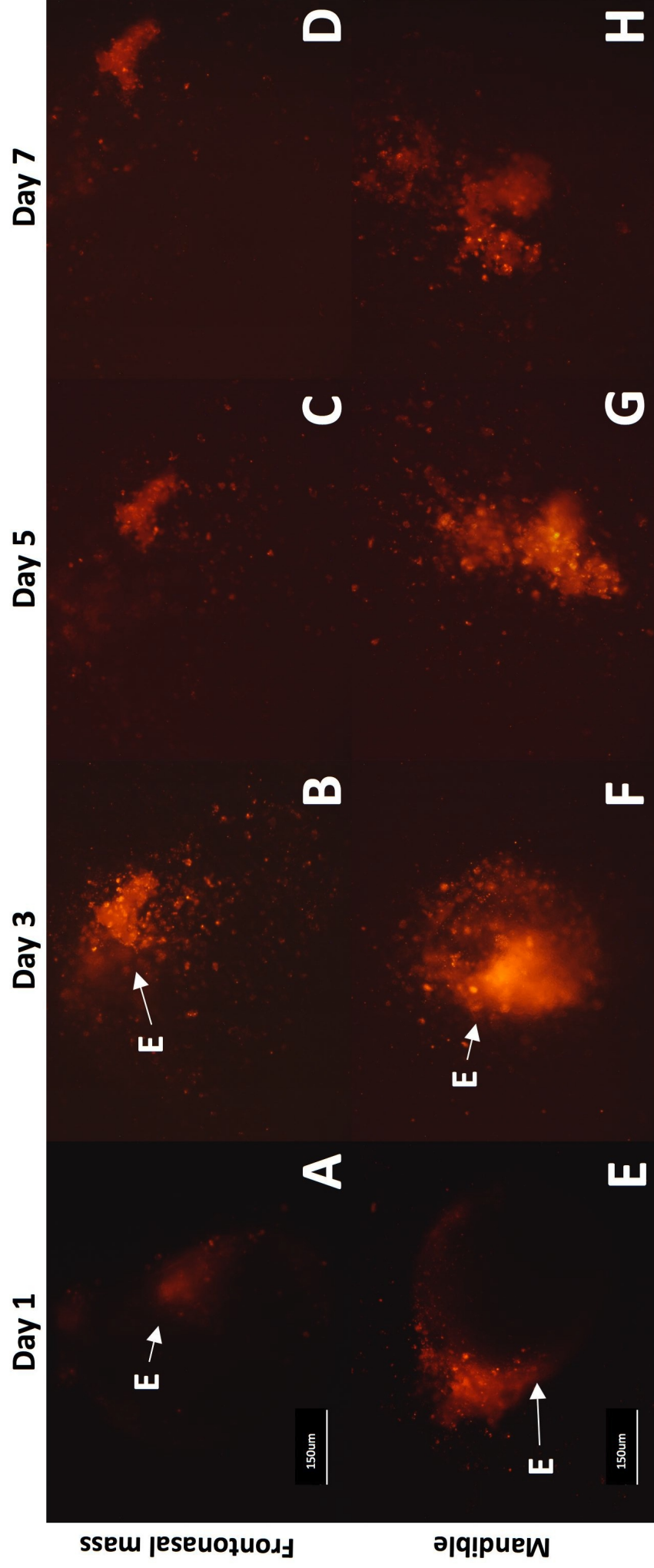


Figure 20: Dil labelled live fluorescent 2D cultures - (A-D) Frontonasal mass explant cultures labelled with Dil. (E-H) Mandible explant cultures labelled with Dil. Fluorescent dye allows cells within the cultures to be located and their subsequent dispersion tracked over the course of 7 days. Cultures most visible at day 3 (B,F) and dye dissipates to become less intense at days 5 (C,G) and 7 (D,H) (Labels: E - explanted tissue)

migrate in a lateral radial manner, the mandible explant culture (Fig.20:E-H) appears to be migrating in a bilateral direction from the original explant, which is concurrent with what has been observed in 2D crystal violet stained cultures, where cells appear to migrate as fibrous extensions from the explant. This bilateral migration pattern may be the result of bilateral orientation of these fibrous structures.

More interestingly with the mandible cultures, when observed under microscope from Day 3 onwards, it was possible to see the labelled cultures contracting rhythmically. Areas of labelled cells were seen to slowly contract towards a central point, remain contracted for a short time and then slowly retract to their original position (see video file in Appendix CD, video shows real time contraction of cells in culture). Figure 21 shows the displacement of labelled cells as the tissue contracts.

This contractility was observed in 4 out of 6 mandible cultures, began at Day 3 and continued until the populations were no longer visible due to the dilution of fluorescence. This adds further evidence towards the argument that the mandible cultures may have some sort of myoblastic population present, as seen with the fibrous outgrowths that are observed extending from the explants in 2D (Fig.7:D,F,H), and with the distortion of the alvetex® after 14 days of culture (Fig.12:B) that is observed with 3D cultures of mandibular tissue.

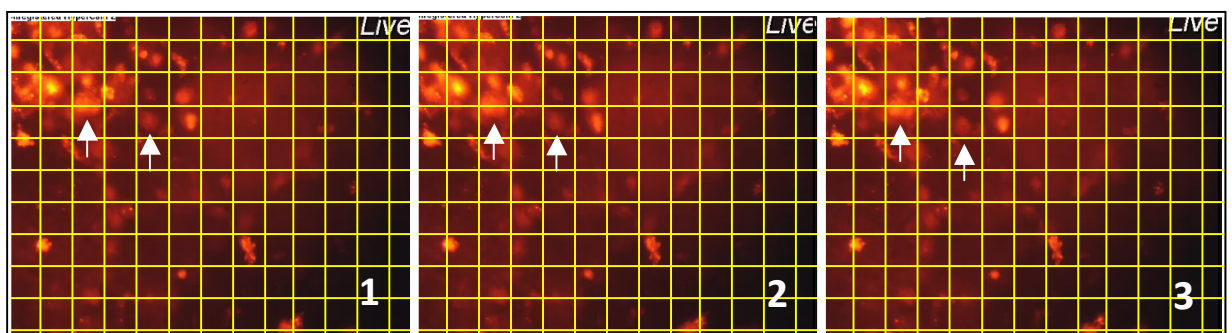


Figure 21: Stills from video capture of contractile behaviour – Three frames with a superimposed yellow grid indicate that cells are moving within the culture. The white arrows indicate two areas of labelled cells that have been displaced as the tissue contracts. As the cells move within the culture (frames 2&3), their positions within the yellow grid can be seen to have shifted from their original positions in frame 1.

Tracking cell migration in a 3D culture system

Figure 22 shows labelled cells in 3D at Day 3. Due to the opaque nature of alvetex® and the use of an inverted fluorescent microscope, it was not possible to initially see the labelled explants as the microscope was observing the underside of the alvetex®, so all resulting images are of the base of the polymer. By Day 3 it was possible to see cells that had migrated further down into the polymer and were beginning to reach the bottom surface of the polymer. This shows that by Day 3 the cells from the explants had migrated far enough from the original explant to be visible from the underside of the polymer, approximately 200um. At this point there was no visible difference observed between the frontonasal mass and the mandible.

2D mandible Dil labelled samples were seen to contract from Day 3, but no such contractions were observed in 3D samples, although when looking at 3D histology of mandibular samples, the distortion of the polymer is only observed in 14 day cultures and not in 7 day cultures.

As with the 2D samples, the fluorescent intensity of the Dil was diluted as the cells migrated and the population expanded, but again due to the opaque nature of the alvetex® this meant that by Day 5 it was no longer possible to locate the cell populations in 3D.

Frontonasal mass

Mandible

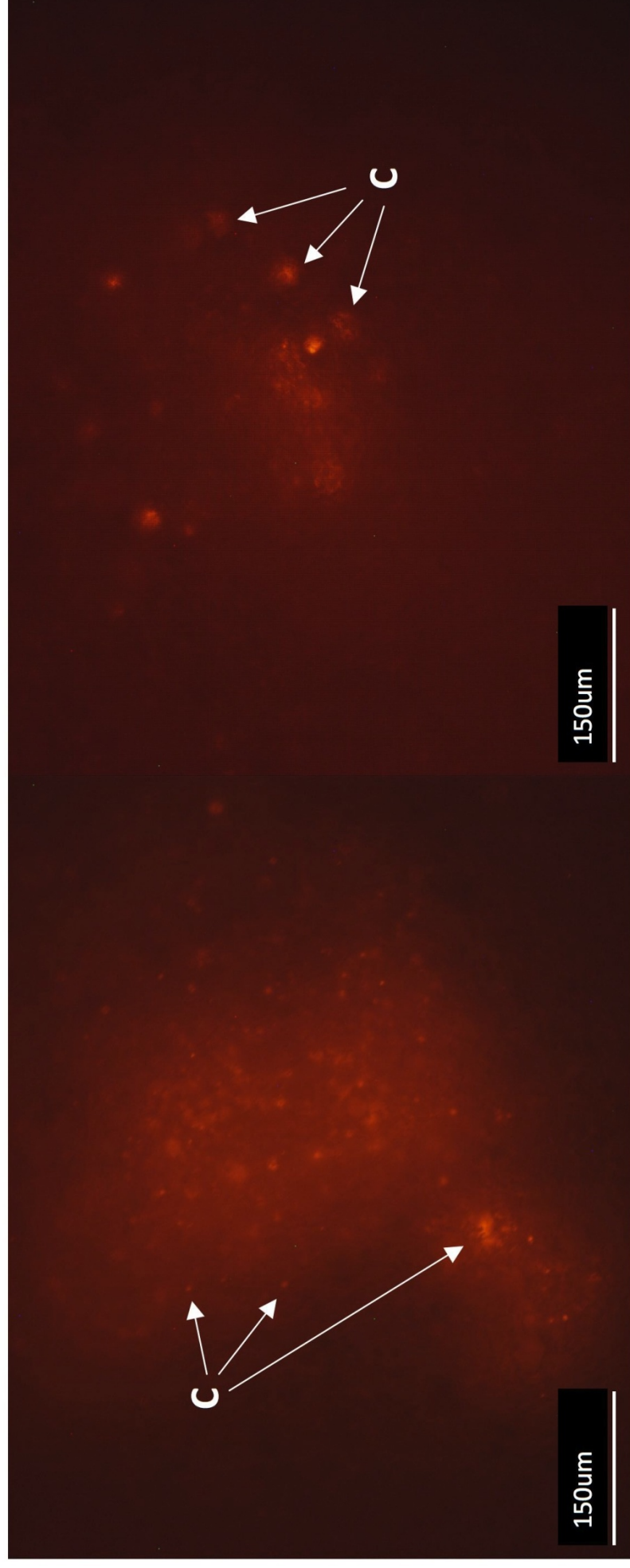


Figure 22: Dil labelled live fluorescent 3D cultures at Day 3 - (A) Frontonasal mass and (B) mandible explants at day 3 in a 3D culture system. As the fluorescent microscope used for this technique is an inverted microscope, the view of labelled cells is from the underside of the polymer disc and as such only labelled cells which have migrated down into the polymer can be located. Cells were difficult to locate at day 1 with little to no fluorescence detectable, at day 3 cells were located within the polymer, but by days 5 and 7, the populations were again difficult to locate with little to no fluorescence, indicating as seen in Fig with the 2D samples that fluorescence seems to be most intense at day 3. (Labels: C - partially labelled cells)

Discussion

Overview of results

The evidence presented above shows that there are distinct differences between the cell behaviour between both tissues of different origins and cells grown in 2D and 3D cell culture systems.

The differences between frontonasal mass and mandible cultures had previously been examined by means of micromass cultures, where frontonasal mass formed sheet-like populations and mandible formed nodular structures. The two tissue types also exhibited different cell behaviours in cultures of explanted mesenchymal tissue. The explant culture method used in this series of experiment has produced results that are both similar and different to previous work with facial processes, with the frontonasal mass producing a sheet-like population that has been previously seen, but the mandible forming fibrous structures rather than the nodular structures of mandibular micromass cultures. These characteristics are more pronounced at 7 days, by the time explanted tissue has been cultured for 14 days, the cell population of the mandible cultures has expanded to include a sheet-like population that resembles that of the frontonasal mass.

3D cultures of mandible and frontonasal mass showed differences in cell density that was observed in both the histological evidence and using cell density measurements. Mandible cultures were found to have a lower modal cell density in 3D culture indicating that they may have a larger amount of chondrogenic material present.

Alcian blue extracts gave further evidence to support the mandible having a larger proportion of chondrogenic material by showing that the mandible had consistently higher levels of Alcian blue staining when compared to the frontonasal mass in both 3D and 2D. Alcian blue extracts also showed that in all cases there was between 4.42 and 8.88-fold increases in Alcian blue staining in 3D cultures when compared to their 2D counterparts, indicating that the alvetex® provided a more permissive environment for chondrogenesis for these mesenchymal explants.

This experiment also showed that it is possible to track living cells in the opaque alvetex® by use of the fluorescent dye Dil, as well as highlighting its use for tracking cell migration in traditional 2D cell culture. In 2D cultures it showed that cells in the mandible cultures were contracting, providing further evidence that a myoblastic population of cells were present. Due to the rhythmic nature of these contractions, it was further speculated that this population of muscle cells were in fact cardiomyoblasts.

Micromass vs. mesenchymal explant culture

Micromass culture is a technique that has been described as method with which to 'study the chondrogenic potential of cells' and has previously been applied to look at chondrogenic patterns of tissue obtained from the facial processes (Wedden et al. 1986). The study showed that the frontonasal mass and the mandible produced different cartilaginous patterns when grown on a 2D substrate using the micromass technique. The frontonasal mass grew as a sheet of cartilaginous material while the mandible grew as discrete nodules, results that I have been able to reproduce successfully. The interpretation of these results relied on comparing the cell behaviours of these two tissue types to the results of micromass experiments that had been conducted with limb buds (Ahrens et al. 1977; Paulsen and Solursh 1988). It was suggested that the homogeneity of the frontonasal mass micromass cultures was due to the cells not yet having received any programming or positional identity information at this stage of development and so all cells within the culture were equally as likely to form cartilage. This was compared to the growth of the progress zone of the limb bud, another developing area where cells remain unspecified, where similar sheets of cartilage were produced using micromass culture. The heterogeneity of the mandible cultures was explained by suggesting that the culture contained different populations of cells that were committed to different lineages by this stage of development (e.g. some to cartilage production, some to connective tissue production) and was compared to cultures of more proximal limb bud mesenchyme, an area of the limb bud that was in a more advanced stage of limb development and also produced nodular cartilage. This

theory can be further substantiated looking at gene profiles of facial processes created by microarray (Buchtova et al. 2010). One of the genes that was expressed more highly in the mandible at an earlier stage was *Msx2*, where a 5.025-fold difference in expression was recorded. It was noted that expression was detected at earlier stages of development in the mandible when compared to the frontonasal mass. Previous work looking at transcription factors associated with the outgrowth of cartilage in the facial primordia show that the morphology of the developing cartilage is influenced by the repression of chondrocyte differentiation by *Msx2* in combination with the promotion of chondrocyte differentiation by *Sox9* (Semba et al. 2000; Takahashi et al. 2001). As *Msx2* is detected in the mandible before the frontonasal mass, this suggests that the mandible begins the process of chondrogenic differentiation and cartilaginous outgrowth at an earlier stage than the frontonasal mass, i.e. chondrogenic patterning is at a more advanced stage in the mandible compared to the frontonasal mass.

Results from my mesenchymal explant experiments seem to add weight to this argument, as the 2D cultures of frontonasal mass clearly demonstrate a very similar cell behaviour, where the cell population that expands from the explanted tissue appears to form a homogenous sheet of cells. This suggests that at this stage of development *in vivo*, the frontonasal mass has not been programmed to produce any particular cartilaginous structure, as it behaves in similar manner when it has either been explanted as a whole piece of tissue or when it is grown in micromass culture using a cell suspension where extracellular matrix and cell contacts are disrupted prior to culture. This means that both culture methods when applied at this stage of development represent the fact the frontonasal mass does not appear to have been provided with any sort of overall pattern of growth to follow and so both methods are equally as representative of the cell behaviour of this tissue *in vivo*. However, the opposite seems to be true for mandibular cultures. The overall appearance of micromass and whole explant cultures are completely different, with micromass forming nodular cartilage structures and the whole explants

producing a population of cartilaginous material and a population of fibrous material.

The fact that these both micromass and explant culture methods produce such different results suggests that perhaps either one or neither of these culture methods are representative of the processes that are occurring *in vivo* if they are capable of producing such varying results.

In the micromass method, the cell suspension that is created breaks any cell contacts and disrupts the extracellular matrix, whereas in the explanted mesenchymal tissue method, all cell contacts and extracellular matrix within that explants remains as it would be *in vivo*. Cell adhesion molecules and extracellular matrix components have been shown to play a significant role in the initiation of chondrogenesis. *In vivo*, that process of condensation can be identified as an aggregation of cells that correlates with an increase in mitotic activity, indicated by an increase in cAMP production. It is thought that TGF-beta and fibronectin regulate N-CAM, a cell adhesion molecule, which brings about the aggregation of cells to form a condensation (Hall and Miyake 2000). In using methods such as the micromass culture which disrupts cell contacts and the extracellular matrix, the normal process of chondrogenesis may itself be disrupted and produce results that are not representative of the processes that occur *in vivo*.

For this reason, the micromass method is likely to be the least representative of the process of chondrogenesis, as it is a method that is designed to assess the chondrogenic potential of a tissue rather than study the cell behaviour inherent to the tissue type in question at a particular stage of development. It relies on breaking down cell contacts and extracellular matrix to induce the production of cartilage. The cell suspension droplet provides an environment of high cell density, allowing the cells within the suspension to aggregate and create areas of even higher cell density which form the condensations that go on to produce cartilage. While this method may not be suitable for creating a representation of cartilage *in vivo* it is a method that can be used to reliably create 2D cultures of cartilaginous material.

The explant method is more likely to be representative of the processes and cell types that are present in the tissue at a particular stage of development. There is no disruption of cell contacts or extracellular matrix within the explanted material itself and so any processes that have begun within the tissue should not be disrupted. The explanted tissue has also been left to grow in culture with no cues to push the population towards producing any particular tissue type or structure and so any behaviour observed is behaviour that is inherent to the tissue type.

However, the explanted tissue has still been removed from its environment, and so any signals that are being provided by the tissue that would usually surround this explanted material are no longer being provided and so this could also alter the cell behaviour. The mesenchyme of the facial processes is greatly influenced by signalling molecules such as BMPs, FGFs and *Shh* that are secreted from various surrounding tissues throughout their development (overview of facial process development, (Santagati and Rijli 2003)). Without the influence of these environmental factors, the growth of this explanted tissue may not be representative of facial process development *in vivo*. This may mean when explants are grown in 3D on an alvetex® substrate, that growth of the culture is an undirected population expansion which does not form any particular cartilaginous structure, but instead simply populates any available space that the alvetex® provides.

Macromolecules, such as collagen II and aggrecan, and adhesion molecules, such as N-CAM and N-cadherin, associated with chondrocyte differentiation have been well characterised. The expression of markers associated with chondrogenesis, in particular interactions between *Sox9* and *Col2a1* (Lefebvre et al. 1997; Zhao et al. 1997; Lefebvre et al. 1998) has been well investigated, and it has been established that *Sox9* protein directly regulates the expression of *Col2a1* by binding to a promoter in the first intron of *Col2a1* (Bell et al. 1997). As these interactions have been well documented both *in vivo* and *in vitro*, it would be interesting to see how the expression of markers and molecules associated with chondrogenesis varied between the two culture methods as this

will almost certainly have an effect on cartilage production within the cultured populations.

A 3D substrate enhances the chondrogenic potential of explanted mesenchymal tissue in comparison to 2D counterparts

Alcian blue staining used in this series of experiments indicates that there is a stark contrast between the amount of chondrogenic material present in 3D cultures and their 2D counterparts (see Figure 18 & Table 1).

The increase in staining ranges from a 4.42 to an 8.88-fold increase in absorbance values of Alcian blue extracts from 3D cultures. These dramatic increases show that the 3D substrate is providing a more permissive environment for the production of cartilaginous material than 2D plasticware. The increase seems to benefit the frontonasal mass cultures the most, with the two highest increases in absorbance being between 2D and 3D counterparts of frontonasal mass cultures. This suggests that the environment increased cartilage production in cell populations derived from frontonasal mass more than cell populations derived from mandible cultures.

It has previously been discussed that the difference between the two tissue types may be due to the tissues being explanted at different stages of chondrogenesis, which allowed the mandible produce more cartilage over the same period of time.

The alvetex® may have allowed a much larger increase in Alcian staining between 2D and 3D frontonasal mass cultures by not only providing a greater surface area for the cells to invade, but also providing a scaffold that allows the cells to immediately form three-dimensional populations. In 2D cultures, three dimensional populations are more difficult to achieve as the cells will initially spread as a monolayer from the explant before beginning to build up the cell layers required for a process such as chondrogenesis to occur. The alvetex® scaffold means that the cells can immediately begin to migrate and interact in a three-dimensional environment that is more akin to that found in vivo. This increase in Alcian blue staining indicates that the provision of a scaffold has improved the capacity of the frontonasal mass to produce cartilaginous

material. As chondrogenesis requires cells to aggregate to form condensations before a region of low cell density can induce cartilage production, the presence of a prefabricated scaffold may aid in the migration of aggregating cells. As the surface area will also have been increased by the scaffold, the size of the area undergoing condensation could also increase, resulting in a larger proportion of chondrogenic material in comparison to 2D counterparts. The combination of an increased substrate surface area and the provision of a prefabricated scaffold may be the reason behind the frontonasal mass cultures having such a dramatic increase in cartilaginous material.

3D mandible cultures also saw an increase in the amount of chondrogenic material present, but not to the same degree as the frontonasal mass. As previously discussed, the mandible explants already appear to contain low cell density chondrogenic material that is invading the polymer. In this case, the increase in the amount of cartilaginous material present in the 3D samples may be due just to the increase in the available surface area that is provided by the alvetex® scaffold, as the explant in this case does not require to undergo the process of aggregation. The increased capacity for forming aggregations in a three-dimensional environment therefore does not apply.

It has previously been discussed that exploring the gene expression profiles of the different facial processes may reveal differences in their ability to produce cartilaginous material. This concept could also be applied to compare the gene expression profiles of 2D and 3D cultures of explanted material before once again comparing these results to what would be expected of these tissues in vivo. As the results presented above show that the 3D cultures seem to be capable of producing more cartilaginous material than their 2D counterparts, it would be interesting to see which of the two culture subsets expression profiles were closer to that seen in vivo. While the 3D may produce more cartilage, is this because the process of chondrogenesis is exaggerated in a 3D environment and the culture is encouraged to produce more cartilaginous material? Previous studies of 3D culture using alvetex® have shown that tissue producing using this method is more similar to tissue found in vivo than its 2D tissue cultures (Schutte et al. 2009), so perhaps gene expression profiles would indicate a

similar result for mesenchymal explants, with 2D cultures of mesenchymal explants proving to be a poor imitator of embryonic tissue in comparison to 3D cultures.

Chondrogenic properties of frontonasal mass and mandible mesenchymal tissue explants

The evidence presented above has shown that there are fundamental differences in cell behaviour between the frontonasal mass and the mandible, both in 2D and 3D cell cultures. The amount of chondrogenic material present in a culture can be determined in a number of ways, two of which are: looking at cell density; and measuring the amount of Alcian blue stain retained by a culture.

In chondrogenic tissue, after cells have aggregated to form a condensation, there is a decrease in cell density in the area of cartilaginous tissue. This area of decreased cell density can be observed in histological sections, as is the case with 2D and 3D cultures, and has also been assessed by calculating the distance between cells and their nearest neighbouring cell in 3D culture.

Observations made about the 2D explants show that there are visible areas of higher and lower cell density within the population of cells that are derived from the explanted material for both tissue types. The absorbance values for extracts of Alcian blue stained samples show that there is Alcian blue staining occurring in 2D cultures. These results can be interpreted to show that there is cartilaginous material present in the 2D cultures. Figure 19 uses Masson's Trichrome histological staining to show that the region of lower cell density may contain collagen, a major constituent of the extracellular matrix in cartilage, adding further weight to the argument that the areas of low cell density observed are regions of chondrogenic tissue.

There is an increase in the absorbance values between 7 and 14 day 2D cultures (see Figure 18 & Table 1), indicating an increase in the quantity of chondrogenic material, which would be expected, as the size of the overall population appears to have expanded between these two time points, a larger population is more likely to have a larger quantity of cartilaginous material.

The histology for 3D cultures at 7 days shows that there are distinct regions of higher and lower cell density in both tissue types. However, the source of the cells that make up these two regions of cell density appears to differ between the two tissue types.

Looking at the 3D mandible cultures (see Figs 11 & 12), the explant itself appears to have an inner cell population of very low cell density, and this population of cells appear to be populating the inner surface of the polymer. However, the cells that comprise the outer layer of the explant also appear to be populating the polymer and forming a region of higher cell density. In this case, the regions of differing cell density are due to the presence of two different populations of cells that populate the polymer at differing cell densities.

Looking at the explanted tissue of the frontonasal mass (see Figs 8 & 10), it appears that although there are three different layers of cells within the explant itself, the cells that are occupying the internal surface of the polymer are cells that closely resemble the inner cell population. As the population that extends from the explant appears to be derived from only one cell type in both the polymer and the equivalent 2D cultures, it can be assumed that within this one population of cells different regions of different cell densities emerge. In chondrogenesis, the area of low cell density where cartilage is produced is usually surrounded by an area of higher cell density called the perichondrium, which acts as a barrier to allow that area of low cell density to remain separate from the surrounding tissue and retain its lower cell density. This is what may be occurring in this frontonasal mass culture. The appearance of this boundary between the regions of differing cell density may differ from the oriented appearance of the perichondrium *in vivo*, but this may be due to the alvetex® restricting the way in which cells can orient themselves within the scaffold.

As the Alcian blue staining shows that mandible cultures have higher absorbance values than frontonasal mass cultures, this must mean that although there are multiple cell types invading the polymer in mandible samples, there is still a larger amount of cartilaginous tissue present in mandible cultures.

If the two tissue types are at different stages of chondrogenesis as discussed earlier, then the mandible explants may have already had a larger amount of

chondrogenic material present at the time of explantation than frontonasal mass explants. If the frontonasal mass has less chondrogenic material to begin with, then the population of cells that are derived from the explant would have to create their own areas of low cell density to allow the process of chondrogenesis to occur, whereas if mandible tissue had already begun to undergo chondrogenesis, then the population simply had to expand into the available polymer substrate as low cell density had already been achieved prior to explantation.

This could also explain the dramatic difference in appearance between the explants themselves. The mandible has only two layers, the inner cell population, which by its low cell density and may be assumed to be chondrogenic material, and the outer layer of cells of much higher cell density. The frontonasal mass has three layers, an outer flattened layer of cells, a layer of low cell density and then an inner population of cells that are more rounded in appearance. It may be that this material has not reached the same stage of chondrogenesis as the mandible at the time of explant, and while the cells have aggregated to form a condensation, they have yet to achieve the low cell density required for cartilage production to commence. Once the explant has been placed into culture, it then has the chance to create an area of lower cell density and begin cartilage production as it invades the substrate below. As the mandible has already passed through this stage of chondrogenesis, it simply has to continue producing cartilage within its substrate and does not have to spend time initiating cartilage production like the frontonasal mass cultures. This would explain why the mandible has a larger value for Alcian absorbance than the frontonasal mass at both 7 days and 14 days, it has the advantage of being ready to produce cartilage as soon as it is placed into culture.

A microarray and qPCR analysis of the *in vivo* gene expression profiles in chick facial processes showed that there were 3094 genes that were expressed in different patterns and in different regions of the developing face of the chick (Buchtova et al. 2010). Of these genes, 56 were identified to have at least a three-fold increase in expression in one region when compared to the expression profile of another region of the face. This shows that the

development of the facial processes is a complicated and variable process with differential gene expression being capable of producing a variety of structures. The study identified the level of variation of marker expression when comparing different facial processes such as the frontonasal mass and the mandible. Using data gathered from this previous experiment, the expression of markers associated with chondrogenesis could be compared between the two facial processes of interest. These expression profiles could then be used to select genes of interest whose expression profiles could be examined in mesenchymal explant cultures. This would allow a comparison to be made between the *in vivo* levels of expression and the levels of expression that are recorded in explanted cultures of mesenchymal material derived from facial processes. Differences in expression would highlight the effects of cell culture on the ability of explanted pieces of mesenchymal tissue to undergo the process of chondrogenesis in comparison to results that would be expected *in vivo*.

Presence of a myoblastic population of cells in mandible cultures

It has been shown throughout this series of experiments that the mandible explant cultures contain at least two different cell populations.

In the 2D explants, mandibular cultures produced long, fibrous extensions from the original explant which are clearly visible at 7 days, and at 14 days they are joined by a secondary population that is similar in appearance to the frontonasal mass culture population.

In the 3D counterparts, looking at the histological sections of 7 day cultures, it appears that while there is an invasion of cells from the low density cell population, which is assumed to be chondrogenic tissue, there is also a secondary invasion of cells from the outer layer of the explant of cell with a different morphology to that of the inner chondrogenic population of cells. Although it is difficult to tell from these histological sections, this secondary invasive population may be the fibrous population that is initially observed in the 2D explants. The whole mount crystal violet staining shows that there is some migration away from the original explant along the surface of the polymer, although there are no specific fibrous shapes visible. Comparing this to the

presence of the secondary invasive population present in the histological sections, this could mean that if this is the fibrous population seen in 2D, that the presence of a 3D scaffold alters the cell behaviour and they try to invade the substrate below the explant rather than extend radially from the explant along the surface.

But as is seen in the 14 day cultures, when looking at the histological sections, this secondary population does not seem to be present at all in the internal surface of the polymer, but there is a high density layer of cells present along the top surface of the polymer that appears to be different to the cells that are present within the polymer itself.

It could be said that this just a layer of the chondrogenic cells that have built up along the top surface of the polymer as the culture has expanded, but the fact that in 14 day mandible cultures the polymer is distorted into a 'U' shape, and the frontonasal mass equivalents have flat, non-distorted polymer sections suggests otherwise.

I suggest that this population is in fact a population of myoblastic cells that have populated the top surface of the polymer. The population of cells that are seen at both 7 and 14 days in 2D culture are fibrous in appearance and reminiscent of myofibrils. The distortion seen in the polymer at 14 days indicates that this top layer of cells is contracting and distorting the top surface of the polymer, forcing the entire disc to bend into a 'U' shape. It forms this 'U' shape because the high density cell layer, that is assumed to be myoblastic, is only present on the top surface of the polymer and not on the bottom surface. This means that only the top surface contracts, hence the 'U' shape.

Although fibrous extensions are not seen in the 3D crystal violet whole mount in situ staining, this may be due to the nature of the alvetex® itself. The polymer is both opaque and porous, and so when a layer of cells is viewed on top of the polymer, the voids in the polymer mean that there is not a plain and uniform background behind the cell layer that would allow the identification of cells as is seen in 2D culture. So these fibrous extensions may be present in the 3D cultures, but the nature of the alvetex® means that it is not possible to see these structures on the surface.

The fact that they are present on the top surface of the polymer rather than the internal surface suggests that the voids of the alvetex® are too restrictive for the growth of a population of myoblasts and their preferred environment is along the top surface of the polymer, so perhaps after the initial invasion of the polymer by the myoblastic population, any significant expansion of a fibrous myoblastic population is restricted to the surface of the polymer.

The distortion of the polymer is only seen at 14 days and not 7 days in 3D cultures, however in 2D cultures the fibrous populations are seen in both the 7 day and 14 day cultures. This may show a fundamental difference in the 3D environment provided by the alvetex®. It may restrict the growth of the myoblastic population so that contraction cannot occur until the population has become more established along the top surface of the polymer, or the rigidity of the polymer may be too great for the smaller 7 day population to distort but the larger 14 day population can overcome the mechanical strength of the polymer. To add further weight to the argument for the presence of a myoblastic population, fluorescent staining of live cell populations revealed that 2D cultures of mandible explants contracted rhythmically when observed under a fluorescent microscope. This was observed in 4 out of 6 mandible cultures that had been treated with Dil and was first seen in Day 3 cultures. Contractions were consistently observed from this point onwards until the end of the experiment when the fluorescent dye was no longer visible under the microscope. This behaviour was only observed in 2D cultures. As it has been said previously, the distortion of the polymer was only observed in 14 day 3D cultures and not in 7 day 3D cultures. The fluorescent Dil experiment was only run for 7 days, as after this point it was no longer possible to locate the 2D cultures due to the dilution of the Dil as the population expanded. 3D populations, due to the opaque nature of the alvetex® were only ever visible at Day 3. However, the observations of contractility in 2D cultures and the distortion of the polymer seen in 14 day 3D cultures can allow the speculation that the high density cell layer that is observed in these 3D cultures at 14 days is in fact a layer of myoblastic cells.

Due to the rhythmic nature of the contractions, it is further speculated that these cultures may contain patches of cardiac muscle rather than skeletal muscle. Contractions of skeletal muscle would give sharper and more sporadic contractions than the smooth and rhythmic contractions that were observed and would also require innervation to produce any contractile behaviour. As the cells were being observed at room temperature at had been removed from the incubating temperature of 37°C, this would likely have slowed down the rate of contraction, so if the cultures were observed at the incubation temperature it might be possible to see the faster, rhythmic contraction that is associated with cardiac muscle.

The presence of cardiac muscle in explanted in mandible tissue may be explained by the presence of pharyngeal mesoderm in explanted mandible mesenchyme.

Pharyngeal mesoderm is a type of mesoderm that is found in the pharyngeal arches (or branchial arches) of the developing embryo (reviewed (Tzahor and Evans 2011). They form part of the core mesoderm of the pharyngeal arches, which includes the mandible. Cells from the pharyngeal mesoderm contribute to tissues that form both heart and pharyngeal muscles (Mjaatvedt et al. 2001; Waldo, Kumiski et al. 2001; Nathan et al. 2008).

If pharyngeal mesoderm makes up the core mesoderm of the mandible, then the pharyngeal mesoderm could possess cells that are capable of differentiating into cardiac progenitor cells. If these cells began to differentiate into cardiac tissue in culture, this would explain the presence of fibrous tissue that rhythmically contracts in cultures of mandibular tissue.

Cardiac muscle development is tightly regulated and restricted within the developing embryo as to ensure that it only develops in the correct locations (Vincent and Buckingham 2010). However, if a population of cells that are capable of producing cardiac muscle is removed from the embryo and hence removed from potential sources of developmental restriction, the cell population may then be capable of producing cardiac muscle.

The presence of cardiac muscle in these cultures could be investigated using methods such as immunofluorescence staining of cultures with chick cardiac

muscle markers, or, again, by looking for at gene expression profiles for mandible cultures and looking for genes associated with cardiomyoblast populations.

The presence of two different populations of cells within the polymer has also highlighted a potential property of the alvetex® itself. The segregation of cell populations in the polymer indicates alvetex® may be capable of sorting cell types in explanted cultures of primary tissue. These results show that after initial invasion of the polymer by two different cell types of differing cell densities, the two cell populations then occupied different regions of the polymer, with the high density population of myoblastic cells being restricted to the upper surface of the polymer and the lower cell density population of chondrogenic cells remaining within the internal surface of the polymer. If the alvetex® is allowing the separation of different cell types in a culture of explanted primary tissue, then it could be applied to other primary tissue types to see if a similar effect is observed. If this is also the case with other tissue types, then this could mean that alvetex® could be used as a sort of chromatographic separation material that can be applied to cultures of living cells, allowing different cell types to be separated from each other in cultures of primary tissue.

Use of fluorescent cell tracking to observe cell cultures in opaque 3D substrates

One of the major problems that is encountered in the use of alvetex® as a 3D cell culture substrate is the opacity of the polymer. It means that when cells are seeded into the scaffold, there is no way of being able to track the live progress of cells within the scaffold. In 2D culture, the culture flask or well can be placed under a microscope and the progress of the culture can be monitored throughout the cell culture process. This is not possible with an opaque 3D substrate. This has proved to be a limiting factor in current work using alvetex®, as the morphology of cultures within the alvetex® can only be observed in fixed histological sections, meaning the morphology of live 3D cultures within the polymer cannot be observed.

In order to overcome this problem I applied Dil to my explanted material, a non-toxic, fluorescent dye that is incorporated into cell membranes that has been

used in previous experiments to track the migration of cells such as neural crest (Serbedzija et al. 1988). It was then possible to view a proportion of the cells that had migrated into the polymer. The microscope used for this experiment was a fluorescent inverted microscope however, which meant that while cells that had migrated down into the polymer scaffold were easily viewed, the actual explant on the surface of the polymer could not be viewed.

This problem could be addressed by using a microscope that viewed the cultures from above rather than below, as it would be possible to see the original explant and to track any migration of cells along the top surface and cells close to the top surface of the polymer. Nevertheless, this technique still allowed the location and observation of live cells within the opaque alvetex®.

In its present form, the protocol developed here can be used to simply ascertain whether or not the cells have invaded the polymer, a useful tool to monitor cell progress in 3D prior to fixation. With further modifications to the cell labelling protocol and experimentation with cameras and dye concentrations this technique could prove invaluable in monitoring cell progress in a system where live cell tracking has previously not been possible. Using equipment such as cell culture chambers or heated stages on microscopes would also mean that it would be possible to view cells at their optimum incubation temperature and so when observing cultures of cells such as cardiac muscle, any observations made about their behaviour would be made within the context of an environment that was more representative of the conditions that they are being cultured under.

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